

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C12N 5/00, 15/00, 15/63	A1	(11) International Publication Number: <b>WO 99/05258</b> (43) International Publication Date: 4 February 1999 (04.02.99)
(21) International Application Number: PCT/US98/14765 (22) International Filing Date: 16 July 1998 (16.07.98) (30) Priority Data: 08/900,510                      25 July 1997 (25.07.97)                      US 09/008,892                      20 January 1998 (20.01.98)                      US (71) Applicants: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 12th floor, 1111 Franklin Street, Oakland, CA 94607-5200 (US). ARCH DEVELOPMENT CORPORATION [US/US]; 1101 East 58th Street, Chicago, IL 60637 (US).		(72) Inventors: GERMAN, Michael, S.; 1543 33rd Avenue, San Francisco, CA 94122 (US). RUBENSTEIN, John, L., R.; 71 Woodland Avenue, San Francisco, CA 94117 (US). SUSSEL, Lori; 2095 15th Avenue, San Francisco, CA 94116 (US). SANDER, Maiké; Apartment 5, 1415 5th Avenue, San Francisco, CA 94122 (US). HARTIGAN-O'CONNOR, Dennis, J.; 1106 Maiden Lane Court #109, Ann Arbor, MI 48105 (US). PEDERSEN, Roger, A.; 166 Madrone Avenue, San Francisco, CA 94127-1318 (US). MENESSES, Juanito, J.; 1295 28th Avenue, San Francisco, CA 94122 (US). BELL, Graeme, I.; 5841 S. Maryland Avenue, MC1028, Chicago, IL 60637 (US). FURUTA, Hiroto; 1-5-40, Horidome-Higashi (apt)-Maison Leviere 101, Wakayama 641 (JP). (74) Agent: FRANCIS, Carol, L.; Bozicevic & Reed LLP, Suite 200, 285 Hamilton Avenue, Palo Alto, CA 94301 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published With international search report.
(54) Title: HUMAN Nkx POLYPEPTIDE-ENCODING NUCLEIC ACIDS AND TRANSGENIC ANIMALS DERIVED THEREFROM (57) Abstract <p>The present invention features a human Nkx-2.2 polypeptide and nucleotide sequences encoding Nkx-2.2 polypeptides. In a particular aspect, the polynucleotide is the nucleotide sequence of SEQ ID NO:1. In addition, the invention features polynucleotide sequences that hybridize under stringent conditions to SEQ ID NO:1. In related aspects the invention features expression vectors and host cells comprising polynucleotides that encode a human Nkx-2.2 polypeptide. The present invention also relates to antibodies that bind specifically to a human Nkx-2.2 polypeptide, and methods for producing human Nkx-2.2 polypeptides. Non-human transgenic animal models are provided for Nkx-2.2 gene function and for Nkx-6.1 gene function, wherein the transgenic animal is characterized by having a defect in Nkx-2.2 gene function or a defect in Nkx-6.1 gene function and by having a decreased number of insulin-producing cells relative to a normal animal of the same species. Where the transgenic animal contains a defect in Nkx-2.2 gene function, the animal is further characterized by a decreased number of serotonin-producing cells relative to a normal animal of the same species. The transgenic animals may be either homozygous or heterozygous for the Nkx-2.2 or Nkx-6.1 defect.</p>		

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## INTERNATIONAL SEARCH REPORT

International application No  
PCT/US98/14765

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	SUSSEL et al. The Nkx-2.2 Homeobox Gene Plays a Critical Role in Islet Cell Development. Experimental and Clinical Endocrinology and Diabetes. 1997, Vol. 105, No. 4, pages A20-A21, see entire document.	17-21 ----- 22-25

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# INTERNATIONAL SEARCH REPORT

International application No  
PCT/US98/14765

## A. CLASSIFICATION OF SUBJECT MATTER US CL .

800/18, 435/455, 462, 463, 69.1, 92.1, 4, 6, 325, 320.1; 424/9.21; 530/350, 536/25.1, 25.5

## HUMAN Nkx POLYPEPTIDE-ENCODING NUCLEIC ACIDS AND TRANSGENIC ANIMALS DERIVED THEREFROM

### STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

5           This invention was made, at least in part, with a government grant from the National Institutes of Health (Grant No. DK41822). Thus, the U.S. government may have certain rights in this invention.

### FIELD OF THE INVENTION

10           The invention relates generally to the field of nucleotide sequences encoding gene products having a role in pancreatic development, neural development, diabetes, depression, and/or obesity, and non-human, transgenic animal models derived therefrom.

### BACKGROUND OF THE INVENTION

15           Diabetes mellitus is the third leading cause of death in the U.S. and the leading cause of blindness, renal failure, and amputation. Diabetes is also a major cause of premature heart attacks and stroke and accounts for 15% of U.S. health care costs. Approximately 5% of Americans, and as many as 20% of those over the age of 65, have diabetes.

20           Diabetes results from the failure of the  $\beta$ -cells in the islets of Langerhans in the endocrine pancreas to produce adequate insulin to meet metabolic needs. Diabetes is categorized into two clinical forms: Type 1 diabetes (or insulin-dependent diabetes) and Type 2 diabetes (or non-insulin-dependent diabetes). Type 1 diabetes is caused by the loss of the insulin-producing  $\beta$ -cells. Type 2 diabetes is a more strongly genetic disease than Type 1 (Zonana & Rimoin, 1976 N. Engl. J. Med. 295:603), usually has its onset alter in life, and accounts for approximately 90% of  
25           diabetes in the U.S. Affected individuals usually have both a decrease in the capacity of the pancreas to produce insulin and a defect in the ability to utilize the insulin (insulin resistance). Obesity causes insulin resistance, and approximately 80% of individuals with Type 2 diabetes are clinically obese (greater than 20% above ideal body weight). Unfortunately, about one-half of the people in the U.S. affected by Type 2 diabetes are unaware that they have the disease. Clinical  
30           symptoms associated with Type 2 diabetes may not become obvious until late in the disease, and the early signs are often misdiagnosed, causing a delay in treatment and increased complications. While the role of genetics in the etiology of type 2 diabetes is clear, the precise genes involved are largely unknown.

35           Depression and obesity can each be associated with a defect in serotonin production, serotonin metabolism, or serotonin-mediated neurotransmission. Serotonin (5-hydroxytryptamine (5HT)) is a biogenic amine that not only functions as a neurotransmitter (Takaki, et al., 1985 J. Neurosciences 5:1769) and as a hormone, (Kravitz, et al., 1980 J. Exp. Biol. 89:159), but also as a mitogen (Nemeck, et al., 1986 Proc. Natl. Acad. Sci. USA 83:674). In its functions as a

neurotransmitter, serotonin modulates many forms of synaptic transmission and is believed to exert a number of effects on neuronal growth during early development. In addition, serotonin is also believed to modulate numerous sensory, motor, and behavioral processes in the mammalian nervous system (see Jacobs, in *Hallucinogens: Neurochemical, Behavioral, and Clinical Perspectives* (eds. Jacobs) 183-202 (Raven, N.Y., 1984), Sleight et al., in *Serotonin Receptor Subtypes: Basic and clinical aspects*, (eds. Peroutka) 211-227 (Wiley-Liss, New York, NY, 1991), Wilkinson et al. in *Serotonin receptor subtypes: Basic and clinical aspects* (eds. Peroutka) 147-210 (Wiley-Liss, New York, NY, 1991)). In the cortex, transmission at serotonergic synapses contributes to affective and perceptual states; these synapses represent a major site of action of psychotropic drugs such as LSD, Jacobs in *Hallucinogens: Neurochemical, Behavioral, and Clinical Perspectives*, Jacobs, Ed. (Raven Press, New York, 1984), pages. 183-202.

The diverse responses elicited by serotonin are mediated through the activation of a large family of receptor subtypes, Tecott et al. 1993 *Curr. Opin. Neurobiol* 3,310-315. The complexity of this signaling system and the paucity of selective drugs have made it difficult to understand development of serotonin-producing cells and to understand the role of serotonin in depression and obesity.

Attempts to understand depression and other serotonin-related disorders have focused upon understanding the development of the brain. Development of the vertebrate forebrain is an elaborate process that gives rise to a variety of essential structures including the cerebral cortex, basal ganglia, hypothalamus and thalamus. Although much is known about these structures and the functions that they perform, very little is understood about the mechanisms that direct their specification, morphogenesis and differentiation. Recently, however, families of candidate regulatory genes with regionally restricted expression in the neuroepithelium of the forebrain have been identified; these gene families are hypothesized to establish positional identity and to control region-specific morphogenesis and histogenesis of the forebrain (Shimamura et al. 1995 *Development*, 121:3923-3933; Rubenstein, et al., 1994 *Science* 266:578-581).

Nkx-2.1 and Nkx-2.2 are two of the earliest known genes to be expressed in the neuroectodermal cells of the forebrain; they are expressed at the onset of neurulation in restricted ventral forebrain domains (Shimamura et al., 1995 *supra*). In addition, expression of these genes is induced by the secreted molecule *sonic hedgehog* (*Shh*), a known axial mesendodermal signaling protein that is responsible for the induction of the ventral neurons of the forebrain (Ericson et al., 1995 *Cell* 81:747-756; Barth and Wilson, 1995, *Development* 121:1755-1768). The early and spatially limited expression of Nkx-2.1 and Nkx-2.2 in response to a primary inductive signal suggests that these molecules provide the initial positional information for specific ventral regions of the developing forebrain.

Nkx-2.2 is a member of a vertebrate gene family that is homologous to the *Drosophila* NK-2 gene, which is expressed in neuroblast precursors in the *Drosophila* head (Kim and Nirenberg, 1989 *Proc. Natl. Acad. Sci. USA*, 86:7716-7720). The NK-2 gene family is characterized by two

regions of homology: the homeobox and a highly conserved sequence downstream of the homeobox.

In addition to its expression in the brain, Nkx-2.2 is also expressed in the pancreas, pancreatic islet  $\beta$  cells, and hamster insulinoma (HIT) cells (Rudnick et al. 1994 Proc. Natl. Acad. Sci. USA 91:12203-12207). Nkx-2.2 expression in pancreas is accompanied by expression of Nkx-6.1, another NK-2-related gene (Rudnick et al. supra). A partial sequence (Price et al. 1992 Neuron 8:241-255) and a full-length sequence of murine Nkx-2.2 (Hartigan and Rubenstein 1996 Gene 168:271-2) have been published. The sequence of human Nkx-6.1 has been published by Inoue et al. (1997) Genomics 40:367-370. The actual function of Nkx-2.2 or Nkx-6.1 as either transcription factors or as developmental regulators was not previously known.

The pathogenesis of diabetes, depression, and obesity, and the links between these diseases, are complex and not well understood. Moreover, the complex nature of these disorders makes their study difficult. Thus, there is a need for an in vivo model for insulin- and serotonin-producing cells for identification of new compounds for treatment of disorders associated with insulin and serotonin production, as well as for development of new therapies to address such disorders (e.g., methods for replacing or enhancing serotonin-producing and/or insulin-producing cells). In addition, there is a need for a method to identify individuals at risk of developing insulin and serotonin production-associated disorders. Finally, there is little known about the development and differentiation of the pancreatic islet cells or key cell types in the central nervous system.

## SUMMARY OF THE INVENTION

The present invention features a human Nkx-2.2 polypeptide and nucleotide sequences encoding Nkx-2.2 polypeptides. In a particular aspect, the polynucleotide is the nucleotide sequence of SEQ ID NO:1, and polynucleotide sequences that hybridize under stringent conditions to SEQ ID NO:1. In related aspects, the invention features expression vectors and host cells comprising polynucleotides that encode a human Nkx-2.2 polypeptide. The present invention also relates to antibodies that bind specifically to a human Nkx-2.2 polypeptide, and methods for producing human Nkx-2.2 polypeptides.

A primary object of the invention is to provide an isolated human Nkx-2.2 polypeptide-encoding polynucleotide for use in expression of human Nkx-2.2 (e.g. in a recombinant host cell) and for use in, for example, identification of human Nkx-2.2 polypeptide binding compounds (especially those compounds that affect human Nkx-2.2 polypeptide-mediated activity).

Another object of the invention is to provide an isolated human Nkx-2.2 polypeptide-encoding polynucleotide for use in generation of non-human transgenic animal models for Nkx-2.2 gene function, wherein the transgenic animal is characterized by having a defect in Nkx-2.2 gene function (and, because Nkx-2.2 acts upstream of Nkx-6.1, a defect in Nkx-6.1 gene function), and by having a decreased number of insulin-producing cells relative to a normal animal of the same species. Such Nkx-2.2 transgenic animals are further characterized by a decreased number of serotonin-producing cells relative to a normal animal of the same species. Another related object of the invention is to provide non-human transgenic mammals that are characterized by excess or ectopic expression of the Nkx-2.2 gene. The transgenic animals may be either homozygous or heterozygous for an Nkx-2.2 or Nkx-6.1 defect.

In another aspect the invention features a method of screening for biologically active agents that modulate Nkx-2.2 function, wherein the method involves the steps of combining a candidate agent with any one of: (a) a mammalian Nkx-2.2 polypeptide; (b) a cell comprising a nucleic acid encoding a mammalian Nkx-2.2 polypeptide; or (c) a non-human transgenic animal model for function of an Nkx-2.2 gene comprising one of: (i) a knockout of an Nkx-2.2 gene; (ii) an exogenous and stably transmitted mammalian Nkx-2.2 gene sequence; or (iii) an Nkx-2.2 promoter sequence operably linked to a reporter gene; and determining the effect of said agent on Nkx-2.2 function.

In yet another aspect, the invention features a method of screening biologically active agents that modulate Nkx-6.1 function, where the method involves the steps of combining a candidate agent with any one of: (a) a mammalian Nkx-6.1 polypeptide; (b) a cell comprising a nucleic acid encoding a mammalian Nkx-6.1 polypeptide, or (c) a non-human transgenic animal model for function of an Nkx-6.1 gene comprising one of: (i) a knockout of an Nkx-6.1 gene; (ii) an exogenous and stably transmitted mammalian Nkx-6.1 gene sequence; or (iii) an Nkx-6.1 promoter sequence operably linked to a reporter gene; and determining the effect of said agent on Nkx-6.1 function.



In still another aspect, the invention features a method for detecting a predisposition to an Nkx-2.2-associated disorder in an individual by analyzing the genomic DNA or mRNA of an individual for the presence of at least one predisposing Nkx-2.2 polymorphism or a sequence linked to a predisposing polymorphism; wherein the presence of said predisposing polymorphism is indicative of an increased susceptibility to an Nkx-2.2 associated disorder. Examples of Nkx-2.2-associated disorders include diabetes, depression, and obesity.

In another aspect, the invention features a method for detecting a predisposition to an Nkx-6.1-associated disorder in an individual by analyzing the genomic DNA or mRNA of an individual for the presence of at least one predisposing Nkx-6.1 polymorphism or a sequence linked to a predisposing polymorphism; wherein the presence of said predisposing polymorphism is indicative of an increased susceptibility to an Nkx-6.1 associated disorder. Examples of Nkx-6.1-associated disorders include diabetes.

These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the invention more fully set forth below.

The invention will now be described in further detail.

#### BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A and 1B show the genomic sequence of human Nkx-2.2 polypeptide.

#### DETAILED DESCRIPTION OF THE INVENTION

Before the present genetic sequences and transgenic animals are described, it is to be understood that this invention is not limited to the particular methodology, protocols, cell lines, vectors and reagents described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a construct" includes a plurality of such constructs and reference to "the Nkx-2.2-encoding nucleic acid" includes reference to one or more Nkx-2.2-encoding nucleic acids and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices and materials are now described.

All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing, for example, the cell lines, constructs, and methodologies that are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

5

Definitions

"Polynucleotide" as used herein refers to an oligonucleotide, nucleotide, and fragments or portions thereof, as well as to peptide nucleic acids (PNA), fragments, portions or antisense molecules thereof, and to DNA or RNA of genomic or synthetic origin which can be single- or double-stranded, and represent the sense or antisense strand. Where "polynucleotide" is used to refer to a specific polynucleotide sequence (e.g. a Nkx-2.2 polypeptide-encoding polynucleotide), "polynucleotide" is meant to encompass polynucleotides that encode a polypeptide that is functionally equivalent to the recited polypeptide, e.g., polynucleotides that are degenerate variants, or polynucleotides that encode biologically active variants or fragments of the recited polypeptide. Similarly, "polypeptide" as used herein refers to an oligopeptide, peptide, or protein. Where "polypeptide" is recited herein to refer to an amino acid sequence of a naturally-occurring protein molecule, "polypeptide" and like terms are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

By "*antisense polynucleotide*" is mean a polynucleotide having a nucleotide sequence complementary to a given polynucleotide sequence (e.g. a polynucleotide sequence encoding an Nkx-2.2 polypeptide) including polynucleotide sequences associated with the transcription or translation of the given polynucleotide sequence (e.g. a promoter of a polynucleotide encoding an Nkx-2.2 polypeptide), where the antisense polynucleotide is capable of hybridizing to an Nkx-2.2 polypeptide-encoding polynucleotide sequence. Of particular interest are antisense polynucleotides capable of inhibiting transcription and/or translation of an Nkx-2.2-encoding polynucleotide either *in vitro* or *in vivo*.

"*Peptide nucleic acid*" as used herein refers to a molecule which comprises an oligomer to which an amino acid residue, such as lysine, and an amino group have been added. These small molecules, also designated anti-gene agents, stop transcript elongation by binding to their complementary (template) strand of nucleic acid (Nielsen et al 1993 Anticancer Drug Des 8:53-63).

As used herein, "*Nkx-2.2 polypeptide*" refers to an amino acid sequence of a recombinant or nonrecombinant polypeptide having an amino acid sequence of i) a native Nkx-2.2 polypeptide, ii) a biologically active fragment of an Nkx-2.2 polypeptide, iii) biologically active polypeptide analogs of an Nkx-2.2 polypeptide, or iv) a biologically active variant of an Nkx-2.2 polypeptide. Nkx-2.2 polypeptides of the invention can be obtained from any species, particularly mammalian, including human, rodenti (e.g., murine or rat), bovine, ovine, porcine, murine, or equine, preferably rat or human, from any source whether natural, synthetic, semi-synthetic or recombinant. "Human Nkx-2.2 polypeptide" refers to the amino acid sequences of isolated human Nkx-2.2 polypeptide obtained from a human, and is meant to include all naturally-occurring allelic variants, and is not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

As used herein, "*antigenic amino acid sequence*" means an amino acid sequence that either alone or in association with a carrier molecule, can elicit an antibody response in a mammal.

A "*variant*" of a human Nkx-2.2 polypeptide is defined as an amino acid sequence that is altered by one or more amino acids. The variant can have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. More rarely, a variant can have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Similar minor variations can also include amino acid deletions or insertions, or both. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without abolishing biological or immunological activity can be found using computer programs well known in the art, for example, DNASTar software.

A "*deletion*" is defined as a change in either amino acid or nucleotide sequence in which one or more amino acid or nucleotide residues, respectively, are absent as compared to an amino acid sequence or nucleotide sequence of a naturally occurring Nkx-2.2 polypeptide.

An "*insertion*" or "*addition*" is that change in an amino acid or nucleotide sequence which has resulted in the addition of one or more amino acid or nucleotide residues, respectively, as compared to an amino acid sequence or nucleotide sequence of a naturally occurring Nkx-2.2 polypeptide.

A "*substitution*" results from the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively as compared to an amino acid sequence or nucleotide sequence of a naturally occurring Nkx-2.2 polypeptide.

"Nkx-2.2 exon 1 polypeptide" and "Nkx-2.2 exon 2 polypeptide" are meant to refer to the amino acid sequences of an isolated polypeptide encoded by the Nkx-2.2 exon 1 and exon 2 sequences, respectively, which sequences may be obtained from any species, particularly mammalian, including human, rodentia (e.g., murine or rat), bovine, ovine, porcine, murine, or equine, preferably rat or human, from any source whether natural, synthetic, semi-synthetic or recombinant. "Human Nkx-2.2 exon 1 polypeptide" and "human Nkx-2.2 exon 2 polypeptide" refer to the amino acid sequences of isolated human Nkx-2.2 exon 1 polypeptide and Nkx-2.2 exon 2 polypeptide, respectively, obtained from a human, and is meant to include all naturally-occurring allelic variants, and is not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule. An exemplary human Nkx-2.2 exon 1 polypeptide is SEQ ID NO:4; an exemplary human Nkx-2.2 exon 2 polypeptide is SEQ ID NO:6.

The term "*biologically active*" refers to human Nkx-2.2 polypeptide having structural, regulatory, or biochemical functions of a naturally occurring Nkx-2.2 polypeptide. Likewise, "*immunologically active*" defines the capability of the natural, recombinant or synthetic human Nkx-2.2 polypeptide, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

The term "*derivative*" as used herein refers to the chemical modification of a nucleic acid encoding a human Nkx-2.2 polypeptide or the encoded human Nkx-2.2 polypeptide. Illustrative of

such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group. A nucleic acid derivative would encode a polypeptide which retains essential biological characteristics of a natural Nkx-2.2 polypeptide.

As used herein the term "*isolated*" is meant to describe a compound of interest (e.g.,  
5 either a polynucleotide or a polypeptide) that is in an environment different from that in which the compound naturally occurs. "Isolated" is meant to include compounds that are within samples that are substantially enriched for the compound of interest and/or in which the compound of interest is partially or substantially purified.

As used herein, the term "*substantially purified*" refers to a compound (e.g., either a  
10 polynucleotide or a polypeptide) that is removed from its natural environment and is at least 60% free, preferably 75% free, and most preferably 90% free from other components with which it is naturally associated.

"Stringency" typically occurs in a range from about  $T_m - 5^\circ\text{C}$  ( $5^\circ\text{C}$  below the  $T_m$  of the probe) to about  $20^\circ\text{C}$  to  $25^\circ\text{C}$  below  $T_m$ . As will be understood by those of skill in the art, a  
15 stringency hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences.

The term "*hybridization*" as used herein shall include "any process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs 1994 Dictionary of Biotechnology, Stockton Press, New York NY). Amplification as carried out in the polymerase  
20 chain reaction technologies is described in Dieffenbach et al. 1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY.

The term "*transgene*" is used herein to describe genetic material which has been or is about to be artificially inserted into the genome of a mammalian, particularly a mammalian cell of a living animal.

By "*Nkx-2.2 associated disorder*" is meant a physiological condition or disease associated  
25 with altered Nkx-2.2 function (e.g., due to aberrant Nkx-2.2 expression or a defect in Nkx-2.2 expression or in the Nkx-2.2 protein). Such Nkx-2.2 associated disorders can include, but are not necessarily limited to, disorders associated with reduced levels of insulin or the ability to utilize insulin (e.g., hyperglycemia, diabetes (e.g., Type 1 and Type 2 diabetes, and the like),  
30 Parkinson's, disorders associated with reduced serotonin production (e.g., depression and obesity), and disorders associated with neural defects (e.g., defects in motor neurons, serotonin-producing neurons, dopamine neurons, and developmental defects in the forebrain, midbrain, hindbrain, and spinal cord).

By "*Nkx-6.1 associated disorder*" is meant a physiological condition or disease associated  
35 with altered Nkx-6.1 function (e.g., due to aberrant Nkx-6.1 expression or a defect in Nkx-6.1 expression or in the Nkx-6.1 protein). Such Nkx-6.1 associated disorders can include, but are not necessarily limited to, disorders associated with reduced levels of insulin or the ability to utilize insulin (e.g., hyperglycemia, diabetes (e.g., Type 1 and Type 2 diabetes, and the like).

Parkinson's, and disorders associated with neural defects (e.g., defects in motor neurons, serotonin-producing neurons, dopamine neurons, and developmental defects in the forebrain, midbrain, hindbrain, and spinal cord)

5 By "*transformation*" is meant a permanent or transient genetic change, preferably a permanent genetic change, induced in a cell following incorporation of new DNA (i.e., DNA exogenous to the cell). Where the cell is a mammalian cell, a permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell.

10 By "*transgenic organism*" is meant a non-human organism (e.g., single-cell organisms (e.g., yeast), mammal, non-mammal (e.g., nematode or *Drosophila*)) having a non-endogenous (i.e., heterologous) nucleic acid sequence present as an extrachromosomal element in a portion of its cells or stably integrated into its germ line DNA.

15 By "*transgenic animal*" is meant a non-human animal, usually a mammal, having a non-endogenous (i.e., heterologous) nucleic acid sequence present as an extrachromosomal element in a portion of its cells or stably integrated into its germ line DNA (i.e., in the genomic sequence of most or all of its cells). Heterologous nucleic acid is introduced into the germ line of such transgenic animals by genetic manipulation of/for example, embryos or embryonic stem cells of the host animal.

20 A "*knock-out*" of a target gene means an alteration in the sequence of the gene that results in a decrease of function of the target gene, preferably such that target gene expression is undetectable or insignificant. A knock-out of an Nkx-2.2 gene or Nkx-6.1 gene means that function of the Nkx-2.2 gene or Nkx-6.1 gene, respectively, has been substantially decreased so that Nks-2.2 or Nkx-6.1 expression is not detectable or only present at insignificant levels. "Knock-out" transgenics of the invention can be transgenic animals having a heterozygous knock-out of the Nkx-2.2 gene, a homozygous knock-out of the Nkx-2.2 gene, heterozygous knock-out of the Nkx-6.1 gene, a homozygous knock-out of the Nkx-6.1 gene, or any combination thereof. "Knock-outs" also include conditional knock-outs, where alteration of the target gene can occur upon, for example, exposure of the animal to a substance that promotes target gene alteration, introduction of an enzyme that promotes recombination at the target gene site (e.g., Cre in the Cre-lox system), or other method for directing the target gene alteration postnatally.

30 A "*knock-in*" of a target gene means an alteration in a host cell genome that results in altered expression (e.g., increased (including ectopic) or decreased expression) of the target gene, e.g., by introduction of an additional copy of the target gene, or by operatively inserting a regulatory sequence that provides for enhanced expression of an endogenous copy of the target gene. "Knock-in" transgenics of the invention can be transgenic animals having a heterozygous knock-in of the Nkx-2.2 gene, a homozygous knock-in of the Nkx-2.2 gene, heterozygous knock-in of the Nkx-6.1 gene, a homozygous knock-in of the Nkx-6.1 gene, or any combination thereof. "Knock-ins" also encompass conditional knock-ins.

By "*subject*" or "*patient*" is meant any subject for which therapy is desired, including humans, cattle, dogs, cats, guinea pigs, rabbits, rats, mice, insects, horses, chickens, and so on. Of particular interest are subjects having an Nkx-2.2- or Nkx-6.1-associated disorder which is amenable to treatment (e.g., to mitigate symptoms associated with the disorder) by expression of either Nkx-2.2- or Nkx-6.1-encoding nucleic acid in a cell of the subject (e.g., by introduction of the Nkx-2.2- or Nkx-6.1-encoding nucleic acid into the subject in vivo, or by implanting Nkx-2.2- or Nkx-6.1-expressing cells into the subject, which cells also produce a hormone that the subject is in need of (e.g., insulin or serotonin)).

By "*construct*" is meant a recombinant nucleic acid, generally recombinant DNA, that has been generated for the purpose of the expression of a specific nucleotide sequence(s), or is to be used in the construction of other recombinant nucleotide sequences.

By "*operably linked*" is meant that a DNA sequence and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

By "*operatively inserted*" is meant that a nucleotide sequence of interest is positioned adjacent a nucleotide sequence that directs transcription and translation of the introduced nucleotide sequence of interest (i.e., facilitates the production of, e.g., a polypeptide encoded by an Nkx-2.2 or Nkx-6.1 sequence).

## Overview of the Invention

The present invention is based upon the identification and isolation of a polynucleotide sequence encoding a human Nkx-2.2 polypeptide, and on the finding that expression of Nkx-2.2 and expression of Nkx-6.1 are linked to both pancreatic and neural development. Accordingly, the present invention encompasses such human Nkx-2.2 polypeptide encoding polynucleotides, as well as human Nkx-2.2 polypeptides.

Transgenic mice having a heterozygous or a homozygous defect in Nkx-2.2 expression show that Nkx-2.2 expression is necessary for development of  $\beta$  cells, the cells responsible for insulin production, which are located in the islets of Langerhans in the pancreas. Furthermore, Nkx-2.2 expression is also essential for development of serotonin-secreting cells in the brain. Nkx-6.1 expression is important for development of pancreatic  $\beta$  cells and may have a role in the function and/or development of motor neurons, serotonin-producing neurons, and dopamine neurons. Nkx-6.1 expression may be detected in the midbrain, hindbrain, and spinal cord.

The invention also encompasses the use of the polynucleotides disclosed herein to facilitate identification and isolation of polynucleotide and polypeptide sequences having homology to a human Nkx-2.2 polypeptide of the invention. The human Nkx-2.2 polypeptides and polynucleotides of the invention are also useful in the identification of human Nkx-2.2 polypeptide-binding compounds, particularly human Nkx-2.2 polypeptide-binding compounds having human Nkx-2.2 polypeptide agonist or antagonist activity. In addition, the human Nkx-2.2

polypeptides and polynucleotides of the invention are useful in the diagnosis, prevention and treatment of disease associated with human Nkx-2.2 polypeptide biological activity

5 The human Nkx-2.2 polypeptide-encoding polynucleotides of the invention can also be used as a molecular probe with which to determine the structure, location, and expression of the human Nkx-2.2 polypeptide and related polypeptides in mammals (including humans) and to investigate potential associations between disease states or clinical disorders and defects or alterations in human Nkx-2.2 polypeptide structure, expression, or function.

#### Nkx-2.2 and Nkx-6.1 Nucleic Acid

10 The terms "Nkx-2.2 gene" and "Nkx-6.1 gene" herein are used generically to designate Nkx-2.2 and Nkx-6.1 genes, respectively, and their alternate forms. "Nkx-2.2 gene" or "Nkx-6.1 gene" is also intended to mean the open reading frame encoding specific Nkx-2.2 or Nkx-6.1 polypeptides, introns, and adjacent 5' and 3' non-coding nucleotide sequences involved in the regulation of expression, up to about 1 kb beyond the coding region, but possibly further in either  
15 direction. The DNA sequences encoding Nkx-2.2 or Nkx-6.1 may be cDNA or genomic DNA or a fragment thereof. The gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into the host.

The term "cDNA" as used herein is intended to include all nucleic acids that share the arrangement of sequence elements found in native mature mRNA species, where sequence  
20 elements are exons and 3' and 5' non-coding regions. Normally mRNA species have contiguous exons, with the intervening introns removed by nuclear RNA splicing, to create a continuous open reading frame encoding the Nkx-2.2 or Nkx-6.1 polypeptide.

Genomic Nkx-2.2 and Nkx-6.1 sequences have non-contiguous open reading frames, where introns interrupt the protein coding regions. A genomic sequence of interest comprises the  
25 nucleic acid present between the initiation codon and the stop codon, as defined in the listed sequences, including all of the introns that are normally present in a native chromosome. It may further include the 3' and 5' untranslated regions found in the mature mRNA. It may further include specific transcriptional and translational regulatory sequences, such as promoters, enhancers, etc., including about 1 kb, but possibly more, of flanking genomic DNA at either the 5' or 3' end of the transcribed region. The genomic DNA may be isolated as a fragment of 100 kbp  
30 or smaller; and substantially free of flanking chromosomal sequence.

The sequence of this 5' region, and further 5' upstream sequences and 3' downstream sequences, may be utilized for promoter elements, including enhancer binding sites, that provide  
35 for expression in tissues where Nkx-2.2 or Nkx-6.1 are expressed. The tissue specific expression is useful for determining the pattern of expression, and for providing promoters that mimic the native pattern of expression. Naturally occurring polymorphisms in the promoter region are useful for determining natural variations in expression, particularly those that may be associated with disease. Alternatively, mutations may be introduced into the promoter region to determine the



effect of altering expression in experimentally defined systems. Methods for the identification of specific DNA motifs involved in the binding of transcriptional factors are known in the art, e.g. sequence similarity to known binding motifs, gel retardation studies, *etc.* For examples, see Blackwell et al. 1995 Mol Med 1:194-205; Mortlock et al. 1996 Genome Res. 6: 327-33; and Joulin and Richard-Foy (1995) Eur J Biochem 232: 620-626.

The regulatory sequences may be used to identify *cis* acting sequences required for transcriptional or translational regulation of Nkx-2.2 or Nkx-6.1 expression, especially in different tissues or stages of development, and to identify *cis* acting sequences and *trans* acting factors that regulate or mediate Nkx-2.2 or Nkx-6.1 expression. Such transcription or translational control regions may be operably linked to an Nkx-2.2 or Nkx-6.1 gene in order to promote expression of wild type or altered Nkx-2.2 or Nkx-6.1 or other proteins of interest in cultured cells, or in embryonic, fetal or adult tissues, and for gene therapy.

The nucleic acid compositions used in the subject invention may encode all or a part of the Nkx-2.2 or Nkx-6.1 polypeptides as appropriate. Fragments may be obtained of the DNA sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction enzyme digestion, by PCR amplification, *etc.* For the most part, DNA fragments will be of at least about ten contiguous nucleotides, usually at least about 15 nt, more usually at least about 18 nt to about 20 nt, more usually at least about 25 nt to about 50 nt. Such small DNA fragments are useful as primers for PCR, hybridization screening, *etc.* Larger DNA fragments, *i.e.* greater than 100 nt are useful for production of the encoded polypeptide. For use in amplification reactions, such as PCR, a pair of primers will be used. The exact composition of the primer sequences is not critical to the invention, but for most applications the primers will hybridize to the subject sequence under stringent conditions, as known in the art. It is preferable to choose a pair of primers that will generate an amplification product of at least about 50 nt, preferably at least about 100 nt. Algorithms for the selection of primer sequences are generally known, and are available in commercial software packages. Amplification primers hybridize to complementary strands of DNA, and will prime towards each other.

The Nkx-2.2 and Nkx-6.1 genes are isolated and obtained in substantial purity, generally as other than an intact mammalian chromosome. Usually, the DNA will be obtained substantially free of other nucleic acid sequences that do not include an Nkx-2.2. or Nkx-6.1 sequence or fragment thereof, generally being at least about 50%, usually at least about 90% pure and are typically "recombinant", *i.e.* flanked by one or more nucleotides with which it is not normally associated on a naturally occurring chromosome.

The DNA sequences are used in a variety of ways. They may be used as probes for identifying homologs of Nkx-2.2 or Nkx-6.1. Mammalian homologs have substantial sequence similarity to one another, *i.e.* at least 75%, usually at least 90%, more usually at least 95% sequence identity. Sequence similarity is calculated based on a reference sequence, which may be a subset of a larger sequence, such as a conserved motif, coding region, flanking region, *etc.*

A reference sequence will usually be at least about 18 nt long, more usually at least about 30 nt long, and may extend to the complete sequence that is being compared. Algorithms for sequence analysis are known in the art, such as BLAST, described in Altschul et al. 1990 J Mol Biol 215:403-10.

5 Nucleic acids having sequence similarity are detected by hybridization under low stringency conditions, for example, at 50°C and 6XSSC (0.9 M sodium chloride/0.09 M sodium citrate) and remain bound when subjected to washing at 55°C in 1XSSC (0.15 M sodium chloride/0.015 M sodium citrate). Sequence identity may be determined by hybridization under  
10 stringent conditions, for example, at 50°C or higher and 0.1XSSC (15 mM sodium chloride/0.15 mM sodium citrate). By using probes, particularly labeled probes of DNA sequences, one can isolate homologous or related genes. The source of homologous genes may be any species, e.g. primate species, particularly human; rodents, such as rats and mice, canines, felines, bovines, ovines, equines, yeast, *Drosophila*, *Caenorhabditis*, etc.

15 The Nkx-2.2- and Nkx-6.1-encoding DNA may also be used to identify expression of the gene in a biological specimen. The manner in which one probes cells for the presence of particular nucleotide sequences, as genomic DNA or RNA, is well established in the literature and does not require elaboration here. mRNA is isolated from a cell sample. mRNA may be amplified by RT-PCR, using reverse transcriptase to form a complementary DNA strand, followed by  
20 polymerase chain reaction amplification using primers specific for the subject DNA sequences. Alternatively, mRNA sample is separated by gel electrophoresis, transferred to a suitable support, e.g. nitrocellulose, nylon, etc., and then probed with a fragment of the subject DNA as a probe. Other techniques, such as oligonucleotide ligation assays, *in situ* hybridizations, and hybridization to DNA probes arrayed on a solid chip may also find use. Detection of mRNA hybridizing to an Nkx-2.2 or Nkx-6.1 sequence is indicative of Nkx-2.2 or Nkx-6.1 gene expression, respectively, in  
25 the sample.

The Nkx-2.2 and Nkx-6.1 nucleic acid sequences may be modified for a number of purposes, particularly where they will be used intracellularly, for example, by being joined to a nucleic acid cleaving agent, e.g. a chelated metal ion, such as iron or chromium for cleavage of the gene; or the like.

30 The sequence of the Nkx-2.2 and Nkx-6.1 loci, including flanking promoter regions and coding regions, may be mutated in various ways known in the art to generate targeted changes in promoter strength, sequence of the encoded protein, etc. The DNA sequence or product of such a mutation will be substantially similar to the sequences provided herein, i.e. will differ by at least one nucleotide or amino acid, respectively, and may differ by at least two but not more than about  
35 ten nucleotides or amino acids. The sequence changes may be substitutions, insertions or deletions. Deletions may further include larger changes, such as deletions of a domain or exon. Other modifications of interest include epitope tagging, e.g. with the FLAG system, HA, etc. For studies of subcellular localization, fusion proteins with green fluorescent proteins (GFP) may be

used. Such mutated genes may be used to study structure-function relationships of Nkx-2.2 and Nkx-6.1 polypeptides, or to alter properties of the proteins that affect their function or regulation. Such modified Nkx-2.2 and Nkx-6.1 sequences can be used to, for example, generate the transgenic animals of the invention.

Techniques for in vitro mutagenesis of cloned genes are known. Examples of protocols for scanning mutations may be found in Gustin et al., 1993 *Biotechniques* 14:22; Barany, 1985 *Gene* 37:111-23; Colicelli et al., 1985 *Mol Gen Genet* 199:537-9; and Prentki et al., 1984 *Gene* 29:303-13. Methods for site specific mutagenesis can be found in Sambrook et al., 1989 *Molecular Cloning: A Laboratory Manual*, CSH Press, pp. 15.3-15.108; Weiner et al., 1993 *Gene* 126:35-41; Sayers et al., 1992 *Biotechniques* 13:592-6; Jones and Winistorfer, 1992 *Biotechniques* 12:528-30; Barton et al., 1990 *Nucleic Acids Res* 18:7349-55; Marotti and Tomich, 1989 *Gene Anal Tech* 6:67-70; and Zhu 1989 *Anal Biochem* 177:120-4.

#### Nkx-2.2 and/or Nkx-6.1 Transgenic Animals

The Nkx-2.2- and Nkx-6.1-encoding nucleic acids can be used to generate genetically modified non-human animals or site specific gene modifications in cell lines. The term "transgenic" is intended to encompass genetically modified animals having a deletion or other knock-out of Nkx-6.1 and/or Nkx-2.2 gene activity, having an exogenous Nkx-2.2 and/or Nkx-6.1 gene that is stably transmitted in the host cells, "knock-in" having altered Nkx-2.2 and/or Nkx-6.1 gene expression, or having an exogenous Nkx-2.2 and/or Nkx-6.1 promoter operably linked to a reporter gene. Of particular interest are homozygous and heterozygous knock-outs of Nkx-2.2, and homozygous and heterozygous knock-outs of Nkx-6.1, as well as transgenics that are heterozygous knock-outs for both Nkx-2.2 and Nkx-6.1. Transgenics that are homozygous knock-outs for both Nkx-2.2 and Nkx-6.1 are likely of less interest due to the lethality of this combination. Transgenic that are heterozygous knock-outs for both Nkx-2.2 and Nkx-6.1 may be susceptible to an Nkx-2.2/6.1-associated disorder (e.g., diabetes, obesity) at a later age.

Transgenic animals may be made through homologous recombination, where the Nkx-2.2 and/or Nkx-6.1 locus is altered. Alternatively, a nucleic acid construct is randomly integrated into the genome. Vectors for stable integration include plasmids, retroviruses and other animal viruses, YACs, and the like. Of interest are transgenic mammals, preferably a mammal from a genus selected from the group consisting of *Mus* (e.g., mice), *Rattus* (e.g., rats), *Oryctolagus* (e.g., rabbits) and *Mesocricetus* (e.g., hamsters). More preferably the animal is a mouse which is defective or contains some other alteration in Nkx-2.2 and/or Nkx-6.1 gene expression or function. Without being held to theory, within the pancreas Nkx-2.2 apparently acts as a transcriptional activator in a cascade pathway upstream of the transcriptional activity of Nkx-6.1. Therefore, alteration of Nkx-2.2 function will affect Nkx-6.1 function (e.g., a Nkx-2.2 knock-out transgenic animal will have no or little Nkx-6.1 activity present in cells normally exhibiting such activity).

A "knock-out" animal is genetically manipulated to substantially reduce or eliminate endogenous Nkx-2.2 function and/or Nkx-6.1 function, preferably such that target gene expression is undetectable or insignificant. Different approaches may be used to achieve the "knock-out". A chromosomal deletion of all or part of the native Nkx-2.2 and/or Nkx-6.1 homolog may be induced. Deletions of the non-coding regions, particularly the promoter region, 3' regulatory sequences, enhancers, or deletions of gene that activate expression of the Nkx-2.2 and/or Nkx-6.1 genes. A functional knock-out may also be achieved by the introduction of an anti-sense construct that blocks expression of the native Nkx-2.2 and/or Nkx-6.1 genes (for example, see Li and Cohen (1996) Cell 85:319-329).

For both Nkx-2.2 and Nkx-6.1, homozygous knock-outs of the endogenous gene results in a dramatic decrease in insulin production as well as severe neural defects. Because transgenic animals having a homozygous knock-out of either the Nkx-2.2 or Nkx-6.1 genes do not survive long after birth (e.g., null Nkx-2.2 mice survive no more than a few days postnatally (e.g., from about 3 days to about 6 days) while null Nkx-6.1 mice survive no more than a few hours postnatally), use of transgenic animals heterozygous for an Nkx-2.2 gene knock-out or an Nkx-6.1 gene knock-out genes, or transgenic animals homozygous for a less severe defect in Nkx-2.2, or Nkx-6.1 (e.g., a defect that is not lethal within a few hours to days after birth) may be useful as well. For example, a defect in Nkx-2.2 function is associated with a decrease in glucokinase expression in the pancreas in homozygous Nkx-2.2 knock-outs (see Examples below). Since glucokinase is the rate-limiting step in  $\beta$  cell glucose sensing, even modest reductions in glucokinase expression due to altered Nkx-2.2 expression (e.g., due to a heterozygous defect in Nkx-2.2) could decrease  $\beta$  cell glucose sensitivity and causes inadequate insulin production and secretion. Moreover, homozygous null Nkx-2.2 transgenics had roughly normal islet amyloid polypeptide (amylin) expression (see the Examples below). Since amyloid deposits of this peptide have been proposed to cause  $\beta$  cell damage and progressive loss of insulin production in type 2 diabetes, a decreased ratio of insulin/amylin production in individuals with decreased Nkx-2.2 may be another contributor to the disease.

Conditional knock-outs of Nkx-2.2 and/or Nkx-6.1 gene function are also included within the present invention. Conditional knock-outs are transgenic animals that exhibit a defect in Nkx-6.1 and/or Nkx-2.2 gene function upon exposure of the animal to a substance that promotes target gene alteration, introduction of an enzyme that promotes recombination at the target gene site (e.g., Cre in the Cre-loxP system), or other method for directing the target gene alteration.

For example, a transgenic animal having a conditional knock-out of Nkx-6.1 and/or Nkx-2.2 gene function can be produced using the Cre-loxP recombination system (see, e.g., Kilby et al. 1993 Trends Genet 9:413-421). Cre is an enzyme that excises the DNA between two recognition sequences, termed loxP. This system can be used in a variety of ways to create conditional knock-outs of Nkx-6.1 and/or Nkx-2.2. For example, two independent transgenic mice can be produced: one transgenic for an Nkx-2.2, or Nkx-6.1 sequence flanked by loxP sites and a

second transgenic for Cre. The Cre transgene can be under the control of an inducible or developmentally regulated promoter (Gu et al. 1993 Cell 73:1155-1164; Gu et al. 1994 Science 265:103-106), or under control of a tissue-specific or cell type-specific promoter (e.g., a pancreas-specific promoter or brain tissue-specific promoter). The Nkx-2.2 or Nkx-6.1 transgenic is then  
5 crossed with the Cre transgenic to produce progeny deficient for the Nkx-2.2/Nkx-6.1 gene only in those cells that expressed Cre during development.

Transgenic animals may be made having an exogenous Nkx-2.2 gene, an exogenous Nkx-6.1 gene, or both an exogenous Nkx-2.2 gene and an exogenous Nkx-6.1 gene. For example, the transgenic animal may comprise a "knock-in" of an Nkx-2.2 gene, such that the host  
10 cell genome contains an alteration that results in altered expression (e.g., increased (including ectopic) or decreased expression) of an Nkx-2.2 gene, e.g., by introduction of an additional copy of the target gene, or by operatively inserting a regulatory sequence that provides for enhanced expression of an endogenous copy of the target gene. "Knock-in" transgenics can be transgenic animals having a heterozygous knock-in of the Nkx-2.2 gene or a homozygous knock-in of the  
15 Nkx-2.2. "Knock-ins" also encompass conditional knock-ins.

The exogenous gene introduced into the host cell genome to produce a transgenic animal is usually either from a different species than the animal host, or is otherwise altered in its coding or non-coding sequence. The introduced gene may be a wild-type gene, naturally occurring polymorphism, or a genetically manipulated sequence, for example those previously described  
20 with deletions, substitutions or insertions in the coding or non-coding regions. The introduced sequence may encode an Nkx-2.2 and/or Nkx-6.1 polypeptide, or may utilize the Nkx-2.2 and/or Nkx-6.1 promoter operably linked to a reporter gene. Where the introduced gene is a coding sequence, it is usually operably linked to a promoter, which may be constitutive or inducible, and other regulatory sequences required for expression in the host animal.

Specific constructs of interest include, but are not limited to, anti-sense Nkx-2.2 and antisense Nkx-6.1, or a ribozyme based on an Nkx-2.2 or Nkx-6.1 sequence, which will block Nkx-2.2 and Nkx-6.1 expression, respectively, as well as expression of dominant negative Nkx-2.2 and Nkx-6.1 mutations, and over-expression of an Nkx-2.2 and Nkx-6.1 gene. A detectable marker, such as *lac Z* may be introduced into the Nkx-2.2 and/or Nkx-6.1 locus, where upregulation of  
30 expression of the corresponding Nkx gene will result in an easily detected change in phenotype. Constructs utilizing a promoter region of the Nkx-2.2 or Nkx-6.1 genes in combination with a reporter gene or with the coding region of Nkx-2.2 or Nkx-6.1 are also of interest. Constructs having a sequence encoding a truncated or altered (e.g., mutated) Nkx-2.2 or Nkx-6.1 are also of interest.

The modified cells or animals are useful in the study of function and regulation of Nkx-2.2 and Nkx-6.1. Such modified cells or animals are also useful in, for example, the study of the function and regulation of genes whose expression is affected by Nkx-2.2 and/or Nkx-6.1, as well as the study of the development of insulin-secreting cells in the pancreas, and the development of  
35

serotonin-secreting cells in the brain. Thus, the transgenic animals of the invention are useful in identifying downstream targets of Nkx-2.2 and/or Nkx-6.1, as such targets may have a role in the phenotypes associated with defects in Nkx-6.1 and/or Nkx-2.2.

Animals may also be used in functional studies, drug screening, *etc.*, e.g. to determine the effect of a candidate drug on islet cell development, on  $\beta$ -cell function and development, on serotonin-secreting cell development, or on symptoms associated with disease or conditions associated with Nkx-2.2 and/or Nkx-6.1 defects (e.g., on symptoms associated with reduced insulin secretion (e.g., such as that associated with a diabetic syndrome, including Type 2 diabetes), symptoms associated with obesity, or on symptoms associated with reduced serotonin secretion (e.g., symptoms associated with depression). A series of small deletions and/or substitutions may be made in the Nkx-2.2 and/or Nkx-6.1 genes to determine the role of different exons in DNA binding, transcriptional regulation, *etc.* By providing expression of Nkx-2.2 and/or Nkx-6.1 protein in cells in which it is otherwise not normally produced (e.g., ectopic expression), one can induce changes in cell behavior. These animals are also useful for exploring models of inheritance of disorders associated with depression, obesity, and/or diabetes, e.g. dominant v. recessive; relative effects of different alleles and synergistic effects between Nkx-2.2 and Nkx-6.1 and other genes elsewhere in the genome.

DNA constructs for homologous recombination will comprise at least a portion of the Nkx-2.2 or Nkx-6.1 gene with the desired genetic modification, and will include regions of homology to the target locus. DNA constructs for random integration need not include regions of homology to mediate recombination. Conveniently, markers for positive and negative selection are included. Methods for generating cells having targeted gene modifications through homologous recombination are known in the art. For various techniques for transfecting mammalian cells, see Keown et al. 1990 *Methods in Enzymology* 185:527-537.

For embryonic stem (ES) cells, an ES cell line may be employed, or embryonic cells may be obtained freshly from a host, e.g. mouse, rat, guinea pig, *etc.* Such cells are grown on an appropriate fibroblast-feeder layer or grown in the presence of appropriate growth factors, such as leukemia inhibiting factor (LIF). When ES cells have been transformed, they may be used to produce transgenic animals. After transformation, the cells are plated onto a feeder layer in an appropriate medium. Cells containing the construct may be detected by employing a selective medium. After sufficient time for colonies to grow, they are picked and analyzed for the occurrence of homologous recombination or integration of the construct. Those colonies that are positive may then be used for embryo manipulation and blastocyst injection. Blastocysts are obtained from 4 to 6 week old superovulated females. The ES cells are trypsinized, and the modified cells are injected into the blastocoel of the blastocyst. After injection, the blastocysts are returned to each uterine horn of pseudopregnant females. Females are then allowed to go to term and the resulting litters screened for mutant cells having the construct. By providing for a different phenotype of the blastocyst and the ES cells, chimeric progeny can be readily detected.

The chimeric animals are screened for the presence of the modified gene. Chimeric animals having the modification (normally chimeric males) are mated with wildtype animals to produce heterozygotes, and the heterozygotes mated to produce homozygotes. If the gene alterations cause lethality at some point in development, tissues or organs can be maintained as allogeneic or congenic grafts or transplants, or in in vitro culture.

Investigation of genetic function may utilize non-mammalian models, particularly using those organisms that are biologically and genetically well-characterized, such as *C. elegans*, *D. melanogaster* and *S. cerevisiae*. For example, transposon (Tc1) insertions in the nematode homolog of an Nkx-2.2 or Nkx-6.1 gene or a promoter region of an Nkx-2.2 or Nkx-6.1 gene may be made. The Nkx-2.2 or Nkx-6.1 gene sequences may be used to knock-out or to complement defined genetic lesions in order to determine the physiological and biochemical pathways involved in function of islet cells and/or serotonin-secreting cells. It is well known that human genes can complement mutations in lower eukaryotic models.

#### Production of Nkx-2.2 and Nkx-6.1 Polypeptides

Nkx-2.2- and Nkx-6.1-encoding nucleic acid may be employed to synthesize full-length Nkx-2.2 and Nkx-6.1 polypeptides or fragments thereof, particularly fragments corresponding to functional domains; DNA binding sites; etc.; and including fusions of the subject polypeptides to other proteins or parts thereof. For expression, an expression cassette may be employed, providing for a transcriptional and translational initiation region, which may be inducible or constitutive, where the coding region is operably linked under the transcriptional control of the transcriptional initiation region, and a transcriptional and translational termination region. Various transcriptional initiation regions may be employed that are functional in the expression host.

The polypeptides may be expressed in prokaryotes or eukaryotes in accordance with conventional ways, depending upon the purpose for expression. For large scale production of the protein, a unicellular organism, such as *E. coli*, *B. subtilis*, *S. cerevisiae*, or cells of a higher organism such as vertebrates, particularly mammals, e.g. COS 7 cells, may be used as the expression host cells. In many situations, it may be desirable to express the Nkx-2.2 and/or Nkx-6.1 genes in mammalian cells, especially where the encoded polypeptides will benefit from native folding and post-translational modifications. Small peptides can also be synthesized in the laboratory.

With the availability of the polypeptides in large amounts, by employing an expression host, the polypeptides may be isolated and purified in accordance with conventional ways. A lysate may be prepared of the expression host and the lysate purified using HPLC, exclusion chromatography, gel electrophoresis, affinity chromatography, or other purification technique. The purified polypeptide will generally be at least about 80% pure, preferably at least about 90% pure, and may be up to and including 100% pure. Pure is intended to mean free of other proteins, as well as cellular debris.

The Nkx-2.2 and Nkx-6.1 polypeptides can be used for the production of antibodies, where short fragments provide for antibodies specific for the particular polypeptide, and larger fragments or the entire protein allow for the production of antibodies over the surface of the polypeptide. Antibodies may be raised to the wild-type or variant forms of Nkx-2.2 and Nkx-6.1. Antibodies may be raised to isolated peptides corresponding to these domains, or to the native protein, e.g. by immunization with cells expressing Nkx-2.2 or Nkx-6.1, immunization with liposomes having Nkx-2.2 or Nkx-6.1 polypeptides inserted in the membrane, etc.

Antibodies are prepared in accordance with conventional ways, where the expressed polypeptide or protein is used as an immunogen, by itself or conjugated to known immunogenic carriers, e.g. KLH, pre-S HBsAg, other viral or eukaryotic proteins, or the like. Various adjuvants may be employed, with a series of injections, as appropriate. For monoclonal antibodies, after one or more booster injections, the spleen is isolated, the lymphocytes immortalized by cell fusion, and then screened for high affinity antibody binding. The immortalized cells, i.e. hybridomas, producing the desired antibodies may then be expanded. For further description, see Monoclonal Antibodies: A Laboratory Manual, Harlow and Lane eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, 1988. If desired, the mRNA encoding the heavy and light chains may be isolated and mutagenized by cloning in *E. coli*, and the heavy and light chains mixed to further enhance the affinity of the antibody. Alternatives to in vivo immunization as a method of raising antibodies include binding to phage "display" libraries, usually in conjunction with in vitro affinity maturation.

#### Isolation of Nkx-2.2- and Nkx-6.1 Allelic Variants and Homologues in Other Species

Other mammalian Nkx-2.2 and Nkx-6.1 genes can be identified and their function characterized using the Nkx-2.2 and/or Nkx-6.1 genes used in the present invention. Other mammalian Nkx-2.2 and Nkx-6.1 genes of interest include, but are not limited to, human, rodent (e.g. murine, or rat), bovine, feline, canine, and the like, particularly human Nkx-2.2 and Nkx-6.1. Methods for identifying, isolating, sequencing, and characterizing an unknown gene based upon its homology to a known gene sequence are well known in the art (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, CSH Press 1989).

#### Drug Screening

The animal models of the invention, as well as *in vitro* methods using the Nkx-2.2 or Nkx-6.1 polypeptides *in vitro*, can be used to identify candidate agents that affect Nkx-2.2 and/or Nkx-6.1 expression or that interact with Nkx-2.2, and/or Nkx-6.1 polypeptides. Agents of interest can include those that enhance, inhibit, regulate, or otherwise affect Nkx-2.2, and/or Nkx-6.1 activity and/or expression. Agents that enhance Nkx-2.2 and/or Nkx-6.1 activity and/or expression can be used to treat or study disorders associated with decreased Nkx-2.2 and/or Nkx-6.1 activity (e.g., diabetes, obesity, depression), while agents that decrease Nkx-2.2, and/or Nkx-6.1 activity and/or



express can be used to treat or study disorders associated with Nkx-2.2 and/or Nkx-6.1 activity (e.g., increased activity, e.g., insulinomas, expression during development). Candidate agents is meant to include synthetic molecules (e.g., small molecule drugs, peptides, or other synthetically produced molecules or compounds, as well as recombinantly produced gene products) as well as naturally-occurring compounds (e.g., polypeptides, endogenous factors present in insulin-producing and/or serotonin-producing cells, hormones, plant extracts, and the like).

#### Drug Screening Assays

Of particular interest in the present invention is the identification of agents that have activity in affecting Nkx-2.2 and/or Nkx-6.1 expression and/or function. Such agents are candidates for development of treatments for, for example, diabetes (especially Type 2 diabetes), depression (in the case of Nkx-2.2), and obesity (in the case of Nkx-2.2). Drug screening identifies agents that provide a replacement or enhancement for Nkx-2.2 and/or Nkx-6.1 function in affected cells. Conversely, agents that reverse or inhibit Nkx-2.2 or Nkx-6.1 function may provide a means to regulate insulin or serotonin production. Of particular interest are screening assays for agents that have a low toxicity for human cells.

The term "agent" as used herein describes any molecule, e.g., protein or pharmaceutical, with the capability of altering or mimicking the physiological function of Nkx-2.2 and/or Nkx-6.1. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e. at zero concentration or below the level of detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including, but not limited to: peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random

chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

#### Screening of Candidate Agents In Vivo

Agents can be screened for their ability to affect Nkx-2.2, and/or Nkx-6.1 expression or function or to mitigate an undesirable phenotype (e.g., a symptom) associated with an alteration in Nkx-2.2 and/or Nkx-6.1 expression or function. In a preferred embodiment, screening of candidate agents is performed in vivo in a transgenic animal described herein. Transgenic animals suitable for use in screening assays include any transgenic animal having an alteration in Nkx-2.2 and/or Nkx-6.1 expression, and can include transgenic animals having a homozygous or heterozygous knockout of an Nkx-2.2 and/or Nkx-6.1 gene, an exogenous and stably transmitted mammalian Nkx-2.2 and/or Nkx-6.1 gene sequence, and a reporter gene composed of an Nkx-2.2 or Nkx-6.1 promoter sequence operably linked to a reporter gene (e.g.,  $\beta$ -galactosidase, CAT, or other gene that can be easily assayed for expression). The transgenic animals can be either homozygous or heterozygous for the genetic alteration and, where a sequence is introduced into the animal's genome for expression, may contain multiple copies of the introduced sequence.

The candidate agent is administered to a non-human, transgenic animal having altered Nkx-2.2, and/or Nkx-6.1 expression, and the effects of the candidate agent determined. The candidate agent can be administered in any manner desired and/or appropriate for delivery of the agent in order to effect a desired result. For example, the candidate agent can be administered by injection (e.g., by injection intravenously, intramuscularly, subcutaneously, or directly into the tissue in which the desired affect is to be achieved), orally, or by any other desirable means. Normally, the in vivo screen will involve a number of animals receiving varying amounts and concentrations of the candidate agent (from no agent to an amount of agent that approaches an upper limit of the amount that can be delivered successfully to the animal), and may include delivery of the agent in different formulation. The agents can be administered singly or can be combined in combinations of two or more, especially where administration of a combination of agents may result in a synergistic effect.

The effect of agent administration upon the transgenic animal can be monitored by assessing Nkx-2.2 and/or Nkx-6.1 function as appropriate (e.g., by examining expression of a reporter or fusion gene), or by assessing a phenotype associated with the Nkx-2.2- or Nkx-6.1 expression. For example, where the transgenic animal used in the screen contains a defect in Nkx-2.2 expression (e.g., due to a knock-out of the gene), the effect of the candidate agent can be assessed by determining levels of hormones produced in the mouse relative to the levels produced in the Nkx-2.2 defective transgenic mouse and/or in wildtype mice (e.g. by assessing levels of insulin, serotonin, and /or glucagon). Where the transgenic animal used in the screen contains a defect in Nkx-6.1 expression (e.g., due to a knock-out of the gene), the effect of the candidate agent can be assessed by determining levels of, for example, insulin produced in the mouse relative to the levels produced in the Nkx-6.1 defective transgenic mouse and/or in wildtype

mice. Methods for assaying insulin, glucagon, and serotonin are well known in the art. Where the candidate agent affects either Nkx-2.2 and/or Nkx-6.1 expression, and/or affects an Nkx-2.2- and/or Nkx-6.1-associated phenotype, in a desired manner, the candidate agent is identified as an agent suitable for use in therapy of an Nkx-2.2- and/or Nkx-6.1-associated disorder.

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#### Screening of Candidate Agents In Vitro

In addition to screening of agents in the transgenic animals of the invention, a wide variety of in vitro assays may be used for this purpose, including labeled in vitro protein-protein binding assays, protein-DNA binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. For example, by providing for the production of large amounts of Nkx-2.2 or Nkx-6.1 protein, one can identify ligands or substrates that bind to, modulate or mimic the action of the proteins. The purified protein may also be used for determination of three-dimensional crystal structure, which can be used for modeling intermolecular interactions, transcriptional regulation, etc.

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The screening assay can be a binding assay, wherein one or more of the molecules may be joined to a label, and the label directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemilumescers, enzymes, specific binding molecules, particles, e.g. magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

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A variety of other reagents may be included in the screening assays described herein. Where the assay is a binding assay, these include reagents like salts, neutral proteins, e.g. albumin, detergents, etc that are used to facilitate optimal protein-protein binding, protein-DNA binding, and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc. may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 1 hours will be sufficient.

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Other assays of interest detect agents that mimic Nkx-2.2 or Nkx-6.1 function. For example, candidate agents are added to a cell that lacks functional Nkx-2.2 and/or Nkx-6.1, and screened for the ability to reproduce Nkx-2.2 and/or Nkx-6.1 activity in a functional assay.

Many mammalian genes have homologs in yeast and lower animals. The study of such homologs' physiological role and interactions with other proteins in vivo or in vitro can facilitate understanding of biological function. In addition to model systems based on genetic complementation, yeast has been shown to be a powerful tool for studying protein-protein interactions through the two hybrid system described in Chien et al. 1991 Proc. Natl. Acad. Sci.

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USA 88 9578-9582. Two-hybrid system analysis is of particular interest for exploring transcriptional activation by Nkx-2.2 and Nkx-6.1 proteins and to identify cDNAs encoding polypeptides that interact with Nkx-2.2 and/or Nkx-6.1

5                   Identified Candidate Agents

The compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host for treatment of a condition attributable to a defect in Nkx-2.2 and/or Nkx-6.1 function (e.g., a disorder associated with reduced insulin levels (e.g., diabetes (Type 1 or Type 2 diabetes, particularly Type 1 diabetes); a disorder associated with  
10       reduced serotonin levels (e.g., depression and/or obesity). The compounds may also be used to enhance Nkx-2.2 or Nkx-6.1 function. The therapeutic agents may be administered in a variety of ways, orally, topically, parenterally e.g. subcutaneously, intraperitoneally, by viral infection, intravascularly, etc. Inhaled treatments are of particular interest. Depending upon the manner of  
15       introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%.  
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The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art  
20       include aqueous media, vegetable and animal oils and fats. Stabilizing Agents, wetting and emulsifying Agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

Pharmacogenetics

25       Pharmacogenetics is the linkage between an individual's genotype and that individual's ability to metabolize or react to a therapeutic agent. Differences in metabolism or target sensitivity can lead to severe toxicity or therapeutic failure by altering the relation between bioactive dose and blood concentration of the drug. In the past few years, numerous studies have established good relationships between polymorphisms in metabolic enzymes or drug targets, and both  
30       response and toxicity. These relationships can be used to individualize therapeutic dose administration.

Genotyping of polymorphic alleles is used to evaluate whether an individual will respond well to a particular therapeutic regimen. The polymorphic sequences are also used in drug screening assays, to determine the dose and specificity of a candidate therapeutic agent. A  
35       candidate Nkx-2.2 or Nkx-6.1 polymorphism is screened with a target therapy to determine whether there is an influence on the effectiveness in treating, for example, diabetes, depression, and/or obesity. Drug screening assays are performed as described above. Typically two or more different sequence polymorphisms are tested for response to a therapy. Therapies for diabetes

currently include replacement therapy via administration of insulin and administration of drugs that increase insulin secretion (sulfonyl ureas) and drugs that reduce insulin resistance (such as troglitazone). Drugs currently used to treat depression include serotonin uptake blockers (e.g. prozac), while drugs currently used to treat obesity include fen-phen.

5           Where a particular sequence polymorphism correlates with differential drug effectiveness, diagnostic screening may be performed. Diagnostic methods have been described in detail in a preceding section. The presence of a particular polymorphism is detected, and used to develop an effective therapeutic strategy for the affected individual.

#### 10       Detection of Nkx-2.2 or Nkx-6.1 Associated Disorders

          Diagnosis of Nkx-2.2 or Nkx-6.1 associated disorders is performed by protein, DNA or RNA sequence and/or hybridization analysis of any convenient sample from a patient, e.g. biopsy material, blood sample, scrapings from cheek, etc. A nucleic acid sample from a patient having a disorder that may be associated with Nkx-2.2 or Nkx-6.1, is analyzed for the presence of a  
15       predisposing polymorphism in Nkx-2.2 or Nkx-6.1. A typical patient genotype will have at least one predisposing mutation on at least one chromosome. The presence of a polymorphic Nkx-2.2 or Nkx-6.1 sequence that affects the activity or expression of the gene product, and confers an increased susceptibility to an Nkx-2.2 or Nkx-6.1 associated disorder (e.g. hyperglycemia, diabetes, depression, or obesity) is considered a predisposing polymorphism. Individuals are  
20       screened by analyzing their DNA or mRNA for the presence of a predisposing polymorphism, as compared to sequence from an unaffected individual(s). Specific sequences of interest include, for example, any polymorphism that is associated with a diabetic syndrome, especially with Type 2 diabetes, or is otherwise associated with diabetes, including, but not limited to, insertions, substitutions and deletions in the coding region sequence, intron sequences that affect splicing, or  
25       promoter or enhancer sequences that affect the activity and expression of the protein.

          Screening may also be based on the functional or antigenic characteristics of the protein. Immunoassays designed to detect predisposing polymorphisms in Nkx-2.2 or Nkx-6.1 proteins may be used in screening. Where many diverse mutations lead to a particular disease phenotype, functional protein assays can be effective screening tools.

30       Biochemical studies may be performed to determine whether a candidate sequence polymorphism in the Nkx-2.2 or Nkx-6.1 coding region or control regions is associated with disease. For example, a change in the promoter or enhancer sequence that affects expression of Nkx-2.2 or Nkx-6.1 may result in predisposition to diabetes, depression, and/or obesity. Expression levels of a candidate variant allele are compared to expression levels of the normal  
35       allele by various methods known in the art. Methods for determining promoter or enhancer strength include quantitation of the expressed natural protein; insertion of the variant control element into a vector with a reporter gene such as  $\beta$ -galactosidase, luciferase, chloramphenicol

acetyltransferase, etc. that provides for convenient quantitation, and the like. The activity of the encoded Nkx-2.2 or Nkx-6.1 protein may be determined by comparison with the wild-type protein.

A number of methods are available for analyzing nucleic acids for the presence of a specific sequence. Where large amounts of DNA are available, genomic DNA is used directly. Alternatively, the region of interest is cloned into a suitable vector and grown in sufficient quantity for analysis. Cells that express Nkx-2.2 or Nkx-6.1 genes, such as pancreatic cells, may be used as a source of mRNA, which may be assayed directly or reverse transcribed into cDNA for analysis. The nucleic acid may be amplified by conventional techniques, such as the polymerase chain reaction (PCR), to provide sufficient amounts for analysis. The use of the polymerase chain reaction is described in Saiki, et al. 1985 Science 239:487; a review of current techniques may be found in Sambrook, et al. Molecular Cloning: A Laboratory Manual, CSH Press 1989, pp.14.2-14.33. Amplification may also be used to determine whether a polymorphism is present, by using a primer that is specific for the polymorphism. Alternatively, various methods are known in the art that utilize oligonucleotide ligation as a means of detecting polymorphisms, for examples see Riley et al. 1990 Nucl. Acid Res. 18:2887-2890; and Delahunty et al. 1996 Am. J. Hum. Genet. 58:1239-1246.

A detectable label may be included in an amplification reaction. Suitable labels include fluorochromes, e.g. fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), 6-carboxy-X-rhodamine (ROX), 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 5-carboxyfluorescein (5-FAM) or N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), radioactive labels, e.g. <sup>32</sup>P, <sup>35</sup>S, <sup>3</sup>H; etc. The label may be a two stage system, where the amplified DNA is conjugated to biotin, haptens, etc. having a high affinity binding partner, e.g. avidin, specific antibodies, etc., where the binding partner is conjugated to a detectable label. The label may be conjugated to one or both of the primers. Alternatively, the pool of nucleotides used in the amplification is labeled, so as to incorporate the label into the amplification product.

The sample nucleic acid, e.g. amplified or cloned fragment, is analyzed by one of a number of methods known in the art. The nucleic acid may be sequenced by dideoxy or other methods, and the sequence of bases compared to either a neutral Nkx-2.2 or Nkx-6.1 sequence (e.g. an Nkx-2.2 or Nkx-6.1 sequence from an unaffected individual). Hybridization with the variant sequence may also be used to determine its presence, by Southern blots, dot blots, etc. The hybridization pattern of a control and variant sequence to an array of oligonucleotide probes immobilized on a solid support, as described in US 5,445,934, or in WO95/35505, may also be used as a means of detecting the presence of variant sequences. Single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), mismatch cleavage detection, and heteroduplex analysis in gel matrices are used to detect conformational changes created by DNA sequence variation as alterations in electrophoretic mobility.

Alternatively, where a polymorphism creates or destroys a recognition site for a restriction endonuclease (restriction fragment length polymorphism, RFLP), the sample is digested with that endonuclease, and the products size fractionated to determine whether the fragment was digested. Fractionation is performed by gel or capillary electrophoresis, particularly acrylamide or agarose gels.

The hybridization pattern of a control and variant sequence to an array of oligonucleotide probes immobilized on a solid support, as described in US 5,445,934, or in WO95/35505, may be used as a means of detecting the presence of variant sequences. In one embodiment of the invention, an array of oligonucleotides are provided, where discrete positions on the array are complementary to at least a portion of mRNA or genomic DNA of either the Nkx-2.2 or Nkx-6.1 locus. Such an array may comprise a series of oligonucleotides, each of which can specifically hybridize to a nucleic acid, e.g. mRNA, cDNA, genomic DNA, etc. from either the Nkx-2.2 or Nkx-6.1 locus. Usually such an array will include at least 2 different polymorphic sequences, i.e. polymorphisms located at unique positions within the locus, usually at least about 5, more usually at least about 10, and may include as many as 50 to 100 different polymorphisms. The oligonucleotide sequence on the array will usually be at least about 12 nt in length, may be the length of the provided polymorphic sequences, or may extend into the flanking regions to generate fragments of 100 to 200 nt in length. For examples of arrays, see Hacia et al. 1996 Nature Genetics 14:441-447; Lockhart et al. 1996 Nature Biotechnol. 14:1675-1680; and De Risi et al. 1996 Nature Genetics 14:457-460.

Antibodies specific for Nkx-2.2 or Nkx-6.1 polymorphisms may be used in screening immunoassays. A reduction or increase in neutral Nkx-2.2 or Nkx-6.1 and/or presence of an Nkx-2.2 disorder associated polymorphism or an Nkx-6.1-disorder associated polymorphism is indicative that the suspected disorder is Nkx-2.2 or Nkx-6.1-associated. A sample is taken from a patient suspected of having an Nkx-2.2- or Nkx-6.1-associated disorder. Samples, as used herein, include tissue biopsies, biological fluids, organ or tissue culture derived fluids, and fluids extracted from physiological tissues, as well as derivatives and fractions of such fluids. The number of cells in a sample will generally be at least about  $10^3$ , usually at least  $10^4$  more usually at least about  $10^5$ . The cells may be dissociated, in the case of solid tissues, or tissue sections may be analyzed. Alternatively a lysate of the cells may be prepared.

Diagnosis may be performed by a number of methods. The different methods all determine the absence or presence or altered amounts of normal or abnormal Nkx-2.2 or Nkx-6.1 in patient cells suspected of having a predisposing polymorphism in Nkx-2.2 or Nkx-6.1. For example, detection may utilize staining of cells or histological sections, performed in accordance with conventional methods. The antibodies of interest are added to the cell sample, and incubated for a period of time sufficient to allow binding to the epitope, usually at least about 10 minutes. The antibody may be labeled with radioisotopes, enzymes, fluorescers, chemiluminescers, or other labels for direct detection. Alternatively, a second stage antibody or reagent is used to

amplify the signal. Such reagents are well known in the art. For example, the primary antibody may be conjugated to biotin, with horseradish peroxidase-conjugated avidin added as a second stage reagent. Final detection uses a substrate that undergoes a color change in the presence of the peroxidase. The absence or presence of antibody binding may be determined by various methods, including flow cytometry of dissociated cells, microscopy, radiography, scintillation counting, etc.

An alternative method for diagnosis depends on the in vitro detection of binding between antibodies and Nkx-2.2 or Nkx-6.1 in a lysate. Measuring the concentration of Nkx-2.2 or Nkx-6.1 binding in a sample or fraction thereof may be accomplished by a variety of specific assays. A conventional sandwich type assay may be used. For example, a sandwich assay may first attach Nkx-2.2 or Nkx-6.1-specific antibodies to an insoluble surface or support. The particular manner of binding is not crucial so long as it is compatible with the reagents and overall methods of the invention. They may be bound to the plates covalently or non-covalently, preferably non-covalently.

The insoluble supports may be any compositions to which polypeptides can be bound, which is readily separated from soluble material, and which is otherwise compatible with the overall method. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports to which the receptor is bound include beads, e.g. magnetic beads, membranes and microtiter plates. These are typically made of glass, plastic (e.g. polystyrene), polysaccharides, nylon or nitrocellulose. Microtiter plates are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples.

Patient sample lysates are then added to separately assayable supports (for example, separate wells of a microtiter plate) containing antibodies. Preferably, a series of standards, containing known concentrations of normal and/or abnormal Nkx-2.2 or Nkx-6.1 is assayed in parallel with the samples or aliquots thereof to serve as controls. Preferably, each sample and standard will be added to multiple wells so that mean values can be obtained for each. The incubation time should be sufficient for binding, generally, from about 0.1 to 3 hr is sufficient. After incubation, the insoluble support is generally washed of non-bound components. Generally, a dilute non-ionic detergent medium at an appropriate pH, generally 7-8, is used as a wash medium. From one to six washes may be employed, with sufficient volume to thoroughly wash non-specifically bound proteins present in the sample.

After washing, a solution containing a second antibody is applied. The antibody will bind Nkx-2.2 or Nkx-6.1 with sufficient specificity such that it can be distinguished from other components present. The second antibodies may be labeled to facilitate direct, or indirect quantification of binding. Examples of labels that permit direct measurement of second receptor binding include radiolabels, such as  $^3\text{H}$  or  $^{125}\text{I}$ , fluorescers, dyes, beads, chemiluminescers, colloidal particles, and the like. Examples of labels which permit indirect measurement of binding



include enzymes where the substrate may provide for a colored or fluorescent product. In a preferred embodiment, the antibodies are labeled with a covalently bound enzyme capable of providing a detectable product signal after addition of suitable substrate. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art. The incubation time should be sufficient for the labeled ligand to bind available molecules. Generally, from about 0.1 to 3 hr is sufficient, usually 1 hr sufficing.

After the second binding step, the insoluble support is again washed free of non-specifically bound material. The signal produced by the bound conjugate is detected by conventional means. Where an enzyme conjugate is used, an appropriate enzyme substrate is provided so a detectable product is formed.

Other immunoassays are known in the art and may find use as diagnostics. Ouchterlony plates provide a simple determination of antibody binding. Western blots may be performed on protein gels or protein spots on filters, using a detection system specific for Nkx-2.2 or Nkx-6.1 as desired, conveniently using a labeling method as described for the sandwich assay.

Other diagnostic assays of interest are based on the functional properties of Nkx-2.2 or Nkx-6.1 proteins. Such assays are particularly useful where a large number of different sequence changes lead to a common phenotype. For example, a functional assay may be based on the transcriptional changes mediated by Nkx-2.2 or Nkx-6.1 gene products. Other assays may, for example, detect conformational changes, size changes resulting from insertions, deletions or truncations, or changes in the subcellular localization of Nkx-2.2 or Nkx-6.1 proteins.

In a protein truncation test, PCR fragments amplified from the Nkx-2.2 or Nkx-6.1 gene or its transcript are used as templates for in vivo transcription/translation reactions to generate protein products. Separation by gel electrophoresis is performed to determine whether the polymorphic gene encodes a truncated protein, where truncations may be associated with a loss of function.

Diagnostic screening may also be performed for polymorphisms that are genetically linked to a predisposition for diabetes, depression, and/or obesity, particularly through the use of microsatellite markers or single nucleotide polymorphisms. Frequently the microsatellite polymorphism itself is not phenotypically expressed, but is linked to sequences that result in a disease predisposition. However, in some cases the microsatellite sequence itself may affect gene expression. Microsatellite linkage analysis may be performed alone, or in combination with direct detection of polymorphisms, as described above. The use of microsatellite markers for genotyping is well documented. For examples, see Mansfield et al. 1994 Genomics 24:225-233; Ziegler et al. 1992 Genomics 14:1026-1031; Dib et al., supra.

Microsatellite loci that are useful in the subject methods have the general formula:

$U(R)_n U'$ , where

U and U' are non-repetitive flanking sequences that uniquely identify the particular locus. R is a repeat motif, and n is the number of repeats. The repeat motif is at least 2 nucleotides in length up to 7, usually 2-4 nucleotides in length. Repeats can be simple or complex. The flanking sequences U and U' uniquely identify the microsatellite locus within the human genome. U and U' are at least about 18 nucleotides in length, and may extend several hundred bases up to about 1 kb on either side of the repeat. Within U and U', sequences are selected for amplification primers. The exact composition of the primer sequences are not critical to the invention, but they must hybridize to the flanking sequences U and U', respectively, under stringent conditions. Criteria for selection of amplification primers are as previously discussed. To maximize the resolution of size differences at the locus, it is preferable to choose a primer sequence that is close to the repeat sequence, such that the total amplification product is between 100-500 nucleotides in length.

The number of repeats at a specific locus, n, is polymorphic in a population, thereby generating individual differences in the length of DNA that lies between the amplification primers. The number will vary from at least 1 repeat to as many as about 100 repeats or more.

The primers are used to amplify the region of genomic DNA that contains the repeats. Conveniently, a detectable label will be included in the amplification reaction, as previously described. Multiplex amplification may be performed in which several sets of primers are combined in the same reaction tube. This is particularly advantageous when limited amounts of sample DNA are available for analysis. Conveniently, each of the sets of primers is labeled with a different fluorochrome.

After amplification, the products are size fractionated. Fractionation may be performed by gel electrophoresis, particularly denaturing acrylamide or agarose gels. A convenient system uses denaturing polyacrylamide gels in combination with an automated DNA sequencer, see Hunkapillar et al. 1991 Science 254:59-74. The automated sequencer is particularly useful with multiplex amplification or pooled products of separate PCR reactions. Capillary electrophoresis may also be used for fractionation. A review of capillary electrophoresis may be found in Landers, et al. 1993 BioTechniques 14:98-111. The size of the amplification product is proportional to the number of repeats (n) that are present at the locus specified by the primers. The size will be polymorphic in the population, and is therefore an allelic marker for that locus.

#### Therapeutic Uses of Nkx-2.2- and Nkx-6.1-Encoding Nucleic Acid

Nkx-2.2 or Nkx-6.1-encoding nucleic acid can be introduced into a cell to accomplish transformation of the cell, preferably stable transformation, and the transformed cell subsequently implanted into a subject having an Nkx-2.2. or Nkx-6.1 disorder characterized by a deficiency in insulin and/or serotonin production, depending upon the tissue into which the transformed cell is implanted. Preferably, the host cell to be transformed and implanted in the subject is derived from the individual who will receive the transplant (e.g., to provide an autologous transplant). Where the transformed cells are to be inserted into the individual, e.g. into the pancreas, liver, abdominal

cavity, etc., the cells into which the nucleic acid is introduced are preferably stem cells capable of developing into  $\beta$  cells within the pancreatic tissue environment, e.g., stem cells derived from gastrointestinal tissue, or cells capable of expression of insulin upon expression of the Nkx-2.2- or Nkx-6.1-encoding nucleic acid. Where the transformed cells are to be transplanted into the individual to provide increased serotonin production, the cells into which the nucleic acid is introduced are preferably a stem cell that is capable of developing into a serotonin-secreting cell in a brain tissue environment, or a cell capable of production of serotonin upon expression of Nkx-2.2- or Nkx-6.1-encoding nucleic acid.

For example, in a subject having Type 1 diabetes, gastrointestinal stem cells can be isolated from the affected subject, the cells transformed with Nkx-2.2-encoding DNA, and the transformed cells implanted in the affected subject to provide for insulin production. Similarly, Nkx-6.1-encoding DNA can be used to transform gastrointestinal stem cells and the transformed cells implanted into the pancreas to provide for development of insulin-producing cells, thereby delivering insulin to the affected subject. In a subject suffering from obesity and/or depression, a cell suitable for implantation in brain tissue is transformed with Nkx-2.2-encoding DNA, transformed cells selected and expanded, and the transformed, Nkx-2.2-expressing cells implanted into the appropriate site in brain tissue of the affected subject to provide for serotonin production.

Introduction of the Nkx-2.2- or Nkx-6.1-encoding nucleic acid into the cell can be accomplished according to methods well known in the art (e.g., through use of electroporation, microinjection, lipofection infection with a recombinant (preferably replication-deficient) virus, and other means well known in the art). Preferably, the Nkx-2.2 or Nkx-6.1-encoding nucleic acid is operably linked to a promoter that facilitates a desired level of Nkx-2.2 or Nkx-6.1 polypeptide expression (e.g., a promoter derived from CMV, SV40, adenovirus, or a tissue-specific or cell type-specific promoter). Transformed cells containing the Nkx-2.2- or Nkx-6.1-encoding nucleic acid can be selected and/or enriched via, for example, expression of a selectable marker gene present in the Nkx-2.2- or Nkx-6.1-encoding construct or that is present on a plasmid that is co-transfected with the Nkx-2.2- or Nkx-6.1-encoding construct. Typically selectable markers provide for resistance to antibiotics such as tetracycline, hygromycin, neomycin, and the like. Other markers can include thymidine kinase and the like.

The ability of the transformed cells to express the Nkx-2.2- or Nkx-6.1-encoding nucleic acid can be assessed by various methods known in the art. For example, Nkx-2.2 or Nkx-6.1 expression can be examined by Northern blot to detect mRNA which hybridizes with a DNA probe derived from the relevant gene. Those cells that express the desired gene can be further isolated and expanded in in vitro culture using methods well known in the art. The host cells selected for transformation with Nkx-2.2- and/or Nkx-6.1-encoding DNA will vary with the purpose of the ex vivo therapy (e.g., insulin production or serotonin production), the site of implantation of the cells,

and other factors that will vary with a variety of factors that will be appreciated by the ordinarily skilled artisan.

Methods for engineering a host cell for expression of a desired gene product(s) and implantation or transplantation of the engineered cells (e.g., ex vivo therapy) are known in the art (see, e.g., Gilbert et al. 1993 "Cell transplantation of genetically altered cells on biodegradable polymer scaffolds in syngeneic rats," *Transplantation* 56:423-427). For example, for expression of a desired gene in exogenous or autologous cells and implantation of the cells for expression of the desired gene product in brain, see, e.g., Martinez-Serrano et al. 1995 "CNS-derived neural progenitor cells for gene transfer of nerve growth factor to the adult rat brain: complete rescue of axotomized cholinergic neurons after transplantation into the septum," *J Neurosci* 15:5668-5680; Taylor et al. 1997 "Widespread engraftment of neural progenitor and stem-like cells throughout the mouse brain," *Transplant Proc* 29:845-847; Snyder et al. 1997 "Potential of neural "stem-like" cells for gene therapy and repair of the degenerating central nervous system," *Adv Neurol* 1997;72:121-132; Snyder et al. 1996 "Gene therapy in neurology," *Curr Opin Pediatr* 1996 8(6):558-568; Kordower et al. 1997 "Dopaminergic transplants in patients with Parkinson's disease: neuroanatomical correlates of clinical recovery," *Exp Neurol* 144:41-46; Lacorazza et al. 1996 "Expression of human beta-hexosaminidase alpha-subunit gene (the gene defect of Tay-Sachs disease) in mouse brains upon engraftment of transduced progenitor cells," *Nat Med* 2:424-429; Martinez-Serrano et al. 1996 "Ex vivo gene transfer of brain-derived neurotrophic factor to the intact rat forebrain: neurotrophic effects on cholinergic neurons," *Eur J Neurosci* 8:727-735; Snyder 1995 "Immortalized neural stem cells: insights into development; prospects for gene therapy and repair," *Proc Assoc Am Physicians* 107:195-204; Tuszynski et al. 1996 "Gene therapy in the adult primate brain: intraparenchymal grafts of cells genetically modified to produce nerve growth factor prevent cholinergic neuronal degeneration," *Gene Ther* 3:305-314; and Karpati et al. 1996 "The principles of gene therapy for the nervous system," *Trends Neurosci* 19:49-54.

For expression of a desired gene in exogenous or autologous cells and implantation of the cells (e.g., islet cells) into pancreas, see, e.g., Docherty 1997 "Gene therapy for diabetes mellitus," *Clin Sci (Colch)* 92:321-330; Hegre et al. 1976 "Transplantation of islet tissue in the rat," *Acta Endocrinol Suppl (Copenh)* 205:257-281; Sandler et al. 1997 "Assessment of insulin secretion in vitro from microencapsulated fetal porcine islet-like cell clusters and rat, mouse, and human pancreatic islets," *Transplantation* 63:1712-1718; Calafiore 1997 "Perspectives in pancreatic and islet cell transplantation for the therapy of IDDM," *Diabetes Care* 20:889-896; Kenyon et al. 1996 "Islet cell transplantation: beyond the paradigms," *Diabetes Metab Rev* 12:361-372; Sandler; Chick et al. 1977 *Science* "Artificial pancreas using living beta cells: effects on glucose homeostasis in diabetic rats," 197:780-782.

After expansion of the transformed cells in vitro, the cells are implanted into the mammalian subject, preferably into the tissue from which the cells were originally derived, by

methods well known in the art. The number of cells implanted is a number of cells sufficient to provide for expression of levels of Nkx-2.2 or Nkx-6.1 sufficient to provide for enhanced levels of insulin or serotonin production. The number cells to be transplanted can be determined based upon such factors as the levels of polypeptide expression achieved *in vitro*, and/or the number of cells that survive implantation. Preferably the cells are implanted in an area of dense vascularization, and in a manner that minimizes evidence of surgery in the subject. The engraftment of the implant of transformed cells is monitored by examining the mammalian subject for classic signs of graft rejection, i.e., inflammation and/or exfoliation at the site of implantation, and fever.

Alternatively, Nkx-2.2- or Nkx-6.1-encoding nucleic acid can be delivered directly to an affected subject to provide for Nkx-2.2. or Nkx-6.1 expression in a target cell (e.g., a pancreatic cell, brain cell, gut cell, liver cell, or other organ cell capable of expressing Nkx-2.2 or Nkx-6.1 and providing production or insulin or serotonin), thereby promoting development of the cell into an insulin-producing cell (e.g., in pancreas) or a serotonin-producing cell (e.g., in brain) or to cure a defect in Nkx-2.2 and/or Nkx-6.1 expression in the subject. Methods for *in vivo* delivery of a nucleic acid of interest for expression in a target cell are known in the art. For example, *in vivo* methods of gene delivery normally employ either a biological means of introducing the DNA into the target cells (e.g., a virus containing the DNA of interest) or a mechanical means to introduce the DNA into the target cells (e.g., direct injection of DNA into the cells, liposome fusion, pneumatic injection using a "gene gun," or introduction of the DNA via a duct of the pancreas). For other methods of introduction of a DNA of interest into a cell *in vivo*, also see Bartlett et al. 1997 "Use of biolistic particle accelerator to introduce genes into isolated islets of Langerhans," Transplant Proc 29:2201-2202; Furth 1997 "Gene transfer by biolistic process," Mol Biotechnol 7:139-143; Gainer et al. 1996 "Successful biolistic transformation of mouse pancreatic islets while preserving cellular function," Transplantation 61:1567-1571; Docherty 1997 "Gene therapy for diabetes mellitus," Clin Sci (Colch) 92:321-330; Maeda et al. 1994 "Gastroenterology 1994 "Adenovirus-mediated transfer of human lipase complementary DNA to the gallbladder," 106:1638-1644.

The amount of DNA and/or the number of infectious viral particles effective to infect the targeted tissue, transform a sufficient number of cells, and provide for production of a desired level of insulin or serotonin can be readily determined based upon such factors as the efficiency of the transformation *in vitro* and the susceptibility of the targeted secretory gland cells to transformation. For example, the amount of DNA injected into the pancreas of a human is, for example, generally from about 1 µg to 750 mg, preferably from about 500 µg to 500 mg, more preferably from about 10 mg to 200 mg, most preferably about 100 mg. Generally, the amounts of DNA can be extrapolated from the amounts of DNA effective for delivery and expression of the desired gene in an animal model. For example, the amount of DNA for delivery in a human is roughly 100 times the amount of DNA effective in a rat.

Regardless of whether the Nkx-2.2. and/or Nkx-6.1-encoding DNA is introduced *in vivo* or *ex vivo*, the DNA (or cells expressing the DNA) can be administered in combination with other genes and other agents. In addition, Nkx-2.2- and/or Nkx-6.1-encoding DNA (or recombinant cells expressing Nkx-2.2. and/or Nkx-6.1 DNA) can be used therapeutically for disorders associated with, for example, a decrease in insulin production and/or serotonin production, but which are not associated with an alteration in Nkx-6.1 and/or Nkx-2.2 function *per se*. For example, an increase in Nkx-2.2 may cause an increase in insulin production in the  $\beta$  cells of an individual that has decreased insulin production from some other cause not related to function of Nkx-6.1 and/or Nkx-2.2.

### EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to carry out the invention and is not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

#### Example 1: Isolation and Sequencing of a Human Nkx-2.2 Polypeptide-Encoding Polynucleotide

The GenBank non-redundant nucleotide sequence database (<http://www.ncbi.nlm.nih.gov>) was searched using the BLAST program for human sequences related to the mouse Nkx2.2 mRNA (GenBank Accession No. U31566). This analysis identified one human sequence, that of a CpG island DNA genomic Mse 1 fragment, clone 60e5, (GenBank Accession No. Z65806) having 84% nucleotide sequence identity with mouse Nkx2.2 mRNA sequence in the region of the initiating methionine codon.

Two primers were prepared based on the sequence of clone 60e5 (Nkx2.2-P1 and Nkx2.2-P2, (see Table 1 for sequences of all primers)) and used to isolate a P1-derived artificial chromosome (PAC) clone containing the human Nkx2.2 gene. PAC DNA pools (Genome Systems, St. Louis, MO) were screened using the polymerase chain reaction (PCR) and primers Nkx2.2-P1 and P2. The PCR conditions were initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec and extension at 72°C for 30 sec, and final extension at 72°C for 10 min. The PCR reaction contained 1 mM MgCl<sub>2</sub> and 5% dimethylsulfoxide in addition to the standard reagents. The PCR products were separated by electrophoresis on a 4% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME) and the 123 bp PCR product defined by the primers Nkx2.2-P1 and P2 was visualized by staining the agarose

gel with ethidium bromide. One PAC clone, 310N11, was identified as containing the human Nkx2.2 gene.

DNA was prepared from PAC 310N11 and digested with the restriction endonucleases EcoRI, HindIII and Pst I. The restriction fragments were separated by electrophoresis on a 1% agarose gel and blotted to a Nylon membrane which was hybridized with <sup>32</sup>P-labeled Nkx2.2-P1 and Nkx2.2-mP1 (see Table 1); this sequence is complementary to mouse Nkx2.2 mRNA in the region of codons 247-252). An approximately 8 kb HindIII fragment hybridized to both primers suggesting that this fragment contained the entire human Nkx2.2 gene. The 8 kb HindIII fragment was subcloned into the vector pBluescript II SK(+) and partially sequenced using primers Nkx2.2-P1, P2, mP1, mP2 and primers based on newly determined human sequence (Table 1) as well as vector specific primers (T7 and T3). The sequences were obtained using a AmpliTaq FS Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Norwalk, CT) and ABI Prism™ 377 DNA Sequencer (ABI, Foster City, CA). The partial sequence of the human Nkx2.2 gene determined in this manner is shown in Figure 1. It has been deposited in the GenBank database with Accession Nos. AF019414 and AF019415.

The chromosomal localization of the human Nkx2.2 was determined by fluorescence in situ hybridization to normal human metaphase chromosomes as described by Rowley et al 1990, Proc Natl Acad Sci USA 87:9358-9362 using biotin-labeled PAC 310N11 DNA as a probe. Hybridization was detected with fluorescein-conjugated avidin (Vector Laboratories, Burlingame, CA) and chromosomes were identified by staining with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). Biotin-labeled PAC 310N11 DNA specifically hybridized only to human chromosome 20. Specific labeling of 20p11-p12 was observed on four (18 cells), three (6 cells) or two (1 cell) chromatids of the chromosome 20 homologues in 25 cells examined. Of 92 signals observed (92 of 100 20p chromatids were labeled), all 92 (100%) were located at 20p11-p12. Of these, 84 (91%) were located at 20p11, 4 (4.5%) were located at the junction of 20p11-p12, and 4 signals (4.5%) were located at 20p12. No background signals were observed at other chromosomal sites. These results localize the human Nkx2.2 gene to chromosome 20, band p11.

The human Nkx2.2 gene consists of two exons which span a region of about 3.5 kbp of human chromosome 20, band p11. The single intron interrupts codon 87. Exon 1 includes the 5'-untranslated region and codons 1-86 and the first nucleotide of codon 87, and exon 2 includes the second and third nucleotides of codon 87, codon 88-273 and the 3'-untranslated region. The amino acid sequence of Nkx2.2 is highly conserved and there is 98% identity between the human and mouse orthologs. There is 93% nucleotide sequence identity between the protein coding regions of the human and mouse Nkx2.2 genes.

Table 1. Sequences of primers used for isolating and sequencing human Nkx2.2 gene

Primer designation	Sequence (5'-3')
<b>Exon 1</b>	
Nkx2.2-P1	ACGAATTGACCAAGTGAAGCTAC (SEQ ID NO:11)
Nkx2.2-P2	CGTTGGTGTCCGGCAGGTCTAAG (SEQ ID NO:12)
Nkx2.2-P5	AACCCGGGCTGCGGCTGCAGGAAT (SEQ ID NO:13)
<b>Exon 2</b>	
Nkx2.2-mP1	AGCTGTACTGGGCGTTGTAC (SEQ ID NO:14)
Nkx2.2-mP2	TGACGAGTCACCGGACAATG (SEQ ID NO:15)
Nkx2.2-P3	GCTCCAGCTCGTAGGTCTGCGCC (SEQ ID NO:16)
Nkx2.2-P4	GCAGTCGCTGCAGCACATGC (SEQ ID NO:17)
Nkx2.2-P6	ATCCAGGGTGCTCCGAGTCTGGTGCA (SEQ ID NO:18)
Nkx2.2-P7	AGTCGAGTTGACTCTCGGCTCCAC (SEQ ID NO:19)
Nkx2.2-P9	GTGGAGCCGAGAGTCAACTCGACT (SEQ ID NO:20)
Nkx2.2-P10	ACGCAGGTCAAGATCTGGTTCCAG (SEQ ID NO:21)

The genomic sequence of human Nkx-2.2 is shown in Figs. 1A and 1B. The human Nkx-2.2 sequence is composed of two exons (SEQ ID NOS:3 and 4) separated by a single intron (illustrated in Fig. as two sequences (SEQ ID NOS: 8 and 9) separated by approximately 1.2 kb of additional intronic sequence). When spliced, exon 1 (SEQ ID NO:3) and exon 2 (SEQ ID NO:4) are joined to form the coding sequence SEQ ID NO:1, which encodes the full-length human Nkx-2.2 polypeptide (SEQ ID NO:2). The last nucleotide of exon 1 and the first two nucleotides of exon two are joined to form a codon encoding a leucine at residue position 87 in the Nkx-2.2 amino acid sequence (SEQ ID NO:2). In addition to the exon and intronic sequences, the sequenced clone included approximately 104 nucleotides of 5' untranslated sequence and about 851 nucleotides of 3' untranslated sequence.

#### Example 2: Heterozygous Nkx-2.2 Knock-Out Transgenic Mice

To determine the function of Nkx-2.2 in development, mice heterozygous for an Nkx-2.2 mutation, as well as mice homozygous for a null mutation of Nkx-2.2 (see Example 2 below) were generated using standard gene targeting techniques. Briefly, an Nkx-2.2 gene targeting vector was constructed by isolating several genomic DNA clones containing the entire Nkx-2.2 gene from a 129J mouse genomic library using PCR primers based on the rat homeobox and NK-2 box (Price et al. 1992 Neuron 8:241-255). Two partially overlapping genomic clones were isolated; each clone contained an approximately 14 kb insert containing the Nkx-2.2 gene in a relatively central position within each clone. These genomic clones were mapped extensively with restriction



enzymes and Southern analysis. This mapping data provided sufficient information for the construction of an Nkx-2.2 gene replacement vector.

5 A null mutation of Nkx-2.2 was generated by replacing all of the coding sequence of Nkx-2.2, including the homeobox region, with a PGK-neo cassette. Approximately 6 kb of 3' and 4 kb of 5' Nkx-2.2 genomic sequences flanked the deletion in order to facilitate homologous recombination events. The recombinant plasmid also contained a herpes simplex virus thymidine kinase (*tk*) gene flanking the genomic DNA to permit selection against nonhomologous recombination events.

10 The Nkx-2.2 targeting DNA vector was electroporated into mouse (129J) embryonic stem (ES) cells using the positive-negative selection strategy for homologous recombination (Mansour, et al. 1988 Nature 336:348-352). Candidate ES clones were screened for homologous recombination by Southern analysis of the genomic DNA. Four clones contained the mutant allele and had undergone a single homologous integration at the Nkx-2.2 locus.

15 Correctly targeted ES cells were injected into C57BL/6J host blastocysts to generate chimeric mice. Chimeric males that transmit the targeted DNA through the germline will be bred to produce F1 mice that are heterozygous for each of the mutant alleles. Inbreeding between the heterozygous mice will be used to produce homozygous mutant animals.

20 Heterozygous Nkx-2.2 knock-out transgenics survived to adulthood, were fertile, and have an apparently normal phenotype in all assays tested. The Nkx-2.2 heterozygous knock-outs may have decreased insulin and insulin mRNA levels compared to wildtype mice. Obese heterozygotes have been observed at 1 year after birth.

#### Example 3: Homozygous Nkx-2.2 Knock-Out Transgenic Mice

Homozygous Nkx-2.2 knock-out transgenic mice were produced by crossing the heterozygous transgenic mice described in Example 1.

25 Homozygous knock-out (null) Nkx-2.2 mice were grossly indistinguishable from their wildtype and heterozygous littermates at birth. However, by the third day after birth, the homozygous mutant animals displayed growth retardation and did not survive longer than six days postnatally. The gross morphology of the homozygous null Nkx-2.2 transgenic mice appeared normal. However, histological analysis revealed a general reduction in islet cell mass; the exocrine cells appeared unaffected. Immunohistochemical analysis showed that there was a remarkable defect in islet cell development. Insulin was undetectable in comparative studies between mutant and wildtype littermates. In addition, the number of cells producing glucagon were reduced, and the level of glucagon per cell was diminished. Glucokinase expression in the pancreas was undetectable. Radioimmunoassays confirmed the immunohistochemical results. Insulin content 30 in the pancreas of Nkx-2.2 null mice was undetectable, and glucagon content was reduced at least 20-fold relative to wildtype mice.

A large population of cells within clusters of the pancreas did not produce any of the four endocrine hormones (insulin, glucagon, somatostatin, and pancreatic polypeptide). Because

expression of pancreatic polypeptide and somatostatin in the pancreas as a whole was normal. These non-endocrine producing cells, which appeared within normal size islets, may be precursors to the glucagon-producing  $\alpha$  or insulin-producing  $\beta$  cells. The null Nkx-2.2 animals still exhibited roughly normal islet amyloid polypeptide (amylin) expression. A summary of the molecular characteristics of islets in the Nkx-2.2 null transgenic animals (as determined by immunohistochemistry) is provided in Table 2 below.

Table 2: Islet Molecular Characteristics in the Nkx-2.2 Mutant — Summary of Immunohistochemistry Results

<b>Endocrine Hormones</b>	
Insulin	undetectable
Glucagon	reduced
Somatostatin	normal
Pancreatic polypeptide	reduced
<b><math>\beta</math> Cell Markers</b>	
Glucokinase	undetectable
Glut2	undetectable
PC1/PC3	normal
Amylin	normal
<b>Transcription Factors</b>	
Pax6	normal/reduced
Pdx1	reduced
Nkx-6.1 (putative transcription factor)	undetectable

In addition to the defects in the pancreas, the homozygous Nkx-2.2 knock-out transgenic mice also exhibited neural defects. A subset of serotonin-producing cells in the brain were decreased in number relative to the number of serotonin-producing cells in wildtype mice. These data show that Nkx-2.2 plays a critical role in islet development, insulin and glucagon synthesis, and in development of serotonin-producing cells in the brain.

#### Example 3: Heterozygous Nkx-6.1 Knock-Out Transgenic Mice

Heterozygous Nkx-6.1 knock-out mice were generated as described in Example 1 except that the gene targeting vector contained Nkx-6.1-encoding DNA rather than Nkx-2.2-encoding DNA. Briefly, an Nkx-6.1 gene targeting vector was constructed by isolating a genomic DNA clones containing the Nkx-6.1 gene from a 129J mouse genomic library using the hamster Nkx-6.1 cDNA sequence as a probe (Rudnick et al. 1994 Proc. Natl. Acad. Sci. USA 91:12203-12207). A single

clone was obtained that included the entire coding region of the Nkx-6.1 gene. When introduced into ES cells as described above, the targeting vector replaced the first exon (which contained the translation start site) with a neomycin resistance gene. Rather than using the tk- selection described in Example 1, ES colonies were simply screened by Southern blot analysis for homologous integration events. The ES cells were then implanted into blastocysts, chimeras identified, and heterozygotes produced as described above.

Heterozygous Nkx-6.1 knock-out transgenics survived to adulthood, were fertile, and have an apparently normal phenotype in all assays tested.

#### Example 4: Homozygous Nkx-6.1 Knock-Out Transgenic Mice

Homozygous Nkx-6.1 knock-out transgenic mice were produced by crossing the heterozygous Nkx-6.1 knock-out transgenic mice described in Example 3.

The pancreas of the homozygous knock-out (null) Nkx-6.1 transgenic mice had almost no  $\beta$ -cells, but other islet cells appeared normal. The null Nkx-6.1 mice died immediately after birth (within about 1 hour) and exhibited severe neurological defects, including decreased movement and defects in motor neurons.

These data show that Nkx-6.1 plays a critical role in islet development, insulin production, and neural development.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

## WHAT IS CLAIMED IS

1. An isolated human Nkx-2.2 polypeptide
2. The human Nkx-2.2 polypeptide of claim 1, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6.
3. An isolated polynucleotide sequence or complement thereof comprising a polynucleotide sequence encoding a human Nkx-2.2 polypeptide of claim 1.
4. The isolated polynucleotide sequence of claim 3 comprising a polynucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.
5. A recombinant expression vector comprising the polynucleotide sequence of claim 3.
6. An isolated recombinant host cell containing the polynucleotide sequence of claim 3.
7. A method for producing the human Nkx-2.2 polypeptide of claim 1, the method comprising the steps of:
  - a) culturing a recombinant host cell containing a human Nkx-2.2 polypeptide-encoding polynucleotide sequence under conditions suitable for the expression of the polypeptide; and
  - b) recovering the polypeptide from the host cell culture.
8. An isolated antibody that specifically binds the human Nkx-2.2 polypeptide of claim 1.
9. A method for identifying a polynucleotide homologous to the polynucleotide of claim 3, the method comprising the steps of:
  - contacting a polynucleotide probe with a test polynucleotide, the probe comprising at least 15 contiguous nucleotides of a polynucleotide sequence encoding a human Nkx-2.2 polypeptide; and
  - detecting hybridization of the probe with the test polynucleotide;wherein detection of hybridization of the probe to the test polynucleotide indicates that the polynucleotide shares sequence homology with the human Nkx-2.2 polypeptide-encoding polynucleotide.
10. An isolated human Nkx-2.2 exon polypeptide
11. The human Nkx-2.2 exon polypeptide of claim 10, comprising an amino acid sequence of SEQ ID NO:4 or SEQ ID NO:6.

12. An isolated polynucleotide sequence or complement thereof comprising a polynucleotide sequence encoding the human Nkx-2.2 exon polypeptide of claim 10.

13. The isolated polynucleotide sequence of claim 12 comprising SEQ ID NO:3, SEQ ID NO:5 or degenerate variants thereof.

14. An isolated polynucleotide sequence comprising at least 15 contiguous nucleotides of a polynucleotide sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, and SEQ ID NO:10.

15. A method for detecting a predisposition to an Nkx-2.2-associated disorder in an individual, the method comprising:

analyzing the genomic DNA or mRNA of an individual for the presence of at least one predisposing Nkx-2.2 polymorphism or a sequence linked to a predisposing polymorphism; wherein the presence of said predisposing polymorphism is indicative of an increased susceptibility to an Nkx-2.2-associated disorder.

16. The method of claim 15, wherein the Nkx-2.2-associated disorder is selected from the group consisting of diabetes, depression, and obesity.

17. A non-human transgenic animal model for Nkx-2.2 or Nkx-6.1 gene function wherein the transgenic animal is characterized by having a defect in Nkx-2.2 or Nkx-6.1 function and having a decreased level of insulin production and decreased number of insulin-producing cells relative to a normal animal of the same species.

18. The transgenic animal of claim 17, wherein the animal is further characterized by a decrease in serotonin levels relative to a normal animal of the same species.

19. The transgenic animal of claim 17, wherein the animal is heterozygous for a defect in Nkx-2.2 or Nkx-6.1 function.

20. The transgenic animal of claim 17, wherein the animal is homozygous for a defect in Nkx-2.2 or Nkx-6.1 function.

21. The transgenic animal of claim 17, wherein the defect in function is due to a knockout of Nkx-2.2 or Nkx-6.1 expression.

22. A method of screening for biologically active agents that modulate Nkx-2.2 or Nkx-6.1 function, the method comprising

combining a candidate agent with any one of

(a) a mammalian Nkx-2.2 polypeptide,

(b) a cell comprising a nucleic acid encoding a mammalian Nkx-2.2 polypeptide, or

(c) a non-human transgenic animal model for function of an Nkx-2.2 or Nkx-6.1 gene comprising one of: (i) a knockout of an Nkx-2.2 or Nkx-6.1 gene; (ii) an exogenous and stably transmitted mammalian Nkx-2.2 or Nkx-6.1 gene sequence; or (iii) an Nkx-2.2 or Nkx-6.1 promoter sequence operably linked to a reporter gene; and

determining the effect of said agent on Nkx-2.2 or Nkx-6.1 function.

23. A method of screening for biologically active agents that affect a phenotype associated with a defect in Nkx-2.2 or Nkx-6.1 function, the method comprising:

combining a candidate agent with a non-human transgenic animal having a defect in Nkx-2.2 or Nkx-6.1 function; and

determining the effect of said agent on a phenotype associated with Nkx-2.2 or Nkx-6.1 function.

24. The method of claim 23, wherein the phenotype is insulin production or serotonin production.

25. A method for altering development of a mammalian cell, the method comprising the steps of:

introducing into a mammalian cell an exogenous polynucleotide, the exogenous polynucleotide encoding a sequence for alteration of function of an Nkx-2.2 gene or an Nkx-6.1 gene;

wherein the introduced exogenous polynucleotide is expressed in the cell, thereby altering function of Nkx-2.2 or Nkx-6.1 in the cell and the cell is altered in development.

26. The method of claim 25, wherein the developmental alteration provides for production of insulin or serotonin by the cell.

Human Nkx2.2 gene  
5' Untranslated sequence (SEQ ID NO:7)

Exon 1  
Met Ser Leu 3  
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TCCTCCGCACTCCGCCCTCCCGCGCGCGGGCAGGGAGGCCACGAAATGACCACTGAGCTACACATTGGCACAATAATTTGGGCTCTCGAAC

Thr Asn Thr Lys Thr Gly Phe Ser Val Lys Asp Ile Leu Asp Leu Pro Asp Thr Asn Asp Glu Gly Ser Val Ala Glu Gly Pro Glu  
ACC AAC ACA AAG ACG GGG TTT TCG GTC AAG GAC ATC TTA GAC CTG CCG GAC ACC AAC GAT GAG GGC TCT GTG GCC GAA GGT CCG GAG

Glu Glu Asn Glu Gly Pro Glu Pro Ala Lys Arg Ala Gly Pro Leu Gly Gln Gly Ala Leu Asp Ala Val Gln Ser Leu Pro Leu Lys Asn 63

Pro Phe Tyr Asp Ser Ser Asp Asn Pro Tyr Thr Arg Trp Leu Ala Ser Thr Glu Gly Leu Cln Tyr Ser L(eu) (SEQ ID NO: 4) 87  
CCC TTC TAC GAC ACC AGC GAC AAC CCG TAC ACC CGC TCG CTG GCC ACC ACC GAG GGC CTT CAG TAC TCC C GTAAGTAGCAAACTTGGCTGCCGACGC  
| (SEQ ID NO: 3)

CTGGTCCCCTCCATTCTTCAGTCGAGCCCGGGTTCGACGCTGGGAGTGAAGGGGAGGGGCCATGTAAGCCCGGAGCCCCCTCACTCGGATCCGTAGCAAGATTTTTTAAACACCCGCGAT

AGGATGCTCTGCCCTCCCTCTTCAAGCCTCCTTAGTTCGGGGAAGAACTTGGCTCTCCAAA: : : : : 1.2 kb : : : : : TCCCCACGCTCTGCCCGCAGGAAACCGCTTAAGGGCG

| Intronic sequence (SEQ ID NO:8)

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87

Exon 2 (L)<sup>eu</sup> His Gly Leu Ala Ala Gly Ala Pro Pro Gln Asp Ser Ser 100  
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| Intronic sequence (SEQ ID NO: 9)

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Amino sequence (294-307)

130

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FIG. 1A

FIG. 1B

FIG. 1

FIG. 1B



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University of Chicago

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# INTERNATIONAL SEARCH REPORT

International application No  
PCT US98/14765

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) C12N 5/00, 15/00, 15/63

US CL Please See Extra Sheet

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/18; 435/455, 462, 463, 69.1, 92.1, 4, 6, 325, 320.1; 424/9.21; 530/350; 536/25.1, 25.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CAPLUS, EMBASE, BIOSIS, WPIDS, APS

search terms: Nkx-2.2, Nkx-6.1, predisposition, diabetes, obesity, depression, transgenic, mouse, mice, knockout, null

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HARTIGAN et al. The cDNA Sequence of Murine Nkx-2.2. Gene. 1996, Vol. 168, pages 271-272, see entire document.	1-7, 9-14, 22
A	MADSEN et al. Development of the Endocrine Pancreas: Lessons from Studies of Pluripotent Islet Cultures. Pancreatic growth and regeneration. Basel: Nora Sarvetnick. 1997, pages 218-230, see especially page 226.	1-26
A	JENSEN et al. mRNA Profiling of Rat Islet Tumors Reveals Nkx 6.1 as a [Beta]-Cell-Specific Homeodomain Transcription Factor. The Journal of Biological Chemistry. 02 August 1996, Vol. 271, No. 31, pages 18749-18758, see entire document.	1-26

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*d* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

30 SEPTEMBER 1998

Date of mailing of the international search report

29 OCT 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

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Authorized officer

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Form PCT/ISA/210 (second sheet)(July 1992)\*